Stomatal densities in *Arabidopsis thaliana*: physiology, growth and inter-genotypic interactions in relation to water availability

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Thesis submitted for the Degree of Doctor of Philosophy

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September 2010

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Abbreviations

The following general abbreviations and terminology are used throughout.

ϕ_{PSII} - Quantum yield of PSII.

Ψ_{leaf} - Leaf water status.

A - is the net photosynthetic rate.

AL - Actinic light.

C_a - Ambient partial pressure of CO_2.

C_c - Partial pressure of CO_2 in the chloroplast at the site of carboxylation.

C_i - Intercellular partial pressure of CO_2.

Col-0 – *Columbia-0* accession.

Col-5 – *Columbia-5* accession.

DAE - Days after emergence.

DAF - Days after flowering (emergence of first flower).

F_0 - Minimal fluorescence in the dark.

F_m^0 - Maximal fluorescence in the dark.

F_t - Steady state fluorescence.

F_m' - Maximal fluorescence in the light.

F_v - Variable fluorescence in the dark.

F_v - Variable fluorescence in the dark.

F_v' - Variable fluorescence in the light.

F_v/F_m^0 - Maximum quantum yield of PSII.

FLP - Four lips gene.

g_m - Mesophyll diffusion conductance.

g_s - Stomatal conductance.

HW - Treatments under well-watered conditions.

K_{leaf} - Leaf hydraulic conductance.

Ler-0 – *Landsberg-0* accession.

LW - Treatments under water-limited conditions.

ML - Measuring light.

NPQ - Non-photochemical quenching.

PAR - Photosynthetically Active Radiation.

PSI - Photosystem I.

PSII - Photosystem II.

PPFD – Photosynthetic photon flux density.

RNE - Relative neighbouring effect.

RubP - Ribulose biphosphate.

sdd1-1 - Stomatal density and distribution 1-1 mutant.

sdd1-2 - Stomatal density and distribution 1-2 mutant.

SDD1 - Stomatal density and distribution gene.

SP - Saturating pulse of light.

TMM - Too many mouths gene.

YDA - Yoda gene.

Abstract

Stomatal densities in *A. thaliana*: physiology, growth and inter-genotypic interactions in relation to water availability

by

Ángel Ferrero-Serrano

Stomatal aperture in plants represents a compromise between the conservation of water and the optimization of CO_2 fixation. As an important anatomical trait, it governs gaseous exchange with the atmosphere, which in turn determines vegetative growth rate and reproductive output and, by implication, competitive interactions amongst plant species. A comprehensive experimental analysis of two genotypes of *Arabidopsis thaliana* was conducted with the objective of assessing the consequences of changes in stomatal density, to leaf photochemistry, photosynthesis and water use, growth and flowering and the implications for intra- and inter-genotypic interactions. The *sdd1-1* mutant of the *SDD1* gene, with a 2.5 fold increase in stomatal density and its wild-type *col-5* were the principal focus of comparative study. Single-leaf measurements of photosynthesis using a Licor-6400 with an incorporated fluorometer showed that the photochemistry of both mutants was similar and that physiological differences between the two could be ascribed to differences in stomatal conductance. Experimentation under controlled conditions of light, watering regime and temperature showed that isolated plants of the wild type with a reduced stomatal density was able to capture the same amount of CO_2 whilst losing less water, under well watered conditions in comparison to the mutant. Assessment of yield –density relationships indicated that differences in water availability (determined by frequency of watering during the period of vegetative growth) determined the intensity of competition in monogenic stands. Inter-genotypic competition under two watering regimes was assessed using two methodological approaches: a partial response surface analysis in mixture of both genotypes and by a target–neighbour comparison at fixed overall density. Genotype *sdd1-2* was more sensitive to inter-genotypic competition than *col-5*, which was dependent on watering regime. At fixed density, inter-genotypic interactions governed root biomass, with the wild-type being less sensitive than the mutant to reduced water supply. Differences in rate of flowering, as a result of competition, generally reflected observed differences in relative vegetative biomass but allometric relationships between aboveground and belowground biomass were more variable under reduced water supply. Phenotypic plasticity in responses to changes in water supply was evident despite the use of controlled growth conditions and specific genotypes.

Acknowledgments

Working on this Ph.D. has been a wonderful and challenging experience, which benefited from the insights and directions of several people.

I would like to express my gratitude first and foremost to my supervisors; Professor A. M. Mortimer and Dr. James Hartwell, whose expertise, encouragement, understanding, and their infinite patience with my particular slow pace, added considerably to my doctoral experience.

Appreciation also goes out to Dr. Meriel Jones as coordinator of the SENSIBLE Marie Curie Host Fellowship; Jean Wood for technical assistance and everyone in Lab G for their support and patience throughout my graduate program.

I must also acknowledge Prof. Michelle Holbrook, Dr. Maciej Zwieniecki and everyone in their labs in the OEB department at the University of Harvard for their support and guidance with the physiological work on this thesis. A very special thanks goes to Dr. Ann Hild of the University of Wyoming, without whose motivation and encouragement during my MS experience I would not have considered a Ph.D in plant biology. Finally, I would also like to thank my parents, Ángel and Cristina, my brother Nacho and my friends for their support and encouragement through these years.

This research would not have been possible without the financial support by a Marie Curie Early Stage Training Fellowship from the European Union under contract numbers IST-2001-33053 and FP6-508861.

'No hay libro tan malo que no tenga algo bueno'

Miguel de Cervantes Saavedra, Don Quijote de la Mancha

Chapter 1. Introduction

1. 1. Overview of the ecology of *Arabidopsis thaliana*

Arabidopsis thaliana (L) Heyhn, commonly known as mouse ear cress or wild thale, belongs to the mustard family (*Brassicaceae*, formerly *Cruciferae*). It was originally named *Arabis thaliana* by Linnaeus in honour of Johanes Thal (1542-1583), author of the first German flora (Napp-Zinn 1969). The genus *Arabidopsis* comprises nine species and eight subspecies (Al-Shehbaz & O'Kane 2002). All the nine species are mainly found in Europe, two of them are also present in Asia and North America, but only *A. thaliana* has a worldwide distribution (Al-Shehbaz & O'Kane 2002). Although *A. thaliana* has been found in the southern hemisphere including Africa, South America and Australia, the literature on *A. thaliana* biogeography concludes that its native range is western Eurasia and its presence overseas is assumed to be caused by human mobility (Shindo, Bernasconi & Hardtke 2007). Post-glacial spread of *A. thaliana* may be the result of an intrinsic high selfing rate, rather than the result of a particular capacity to adapt to new environments. Mitchell-Olds (2001) and Sharbel et al (2000) argued that *A. thaliana* colonization of northern and central Europe may have started from populations in the Iberian Peninsula and Central Asia during the Pleistocene. Correlation between geographical distribution and gradients of temperature and precipitation has been found and it is argued that *A. thaliana* distribution in Northern Europe is limited by low temperatures, while southern distribution may be limited by high temperature and low precipitation (Hoffmann 2002; Hoffmann 2005).

A. thaliana shows a wide variety of genetic and plastic variation among the different wild-type lines. These lines are often referred as 'ecotypes' but are now more commonly referred to by the more neutral term of 'accessions'. The progressive increase of natural variation in *A. thaliana* is due to the wide distribution of natural populations (Shindo, Bernasconi & Hardtke 2007). Hundreds of accessions from natural populations collected from diverse worldwide locations are currently available from public sources (Koornneef, Alonso-Blanco & Vreugdenhil 2004). By January 2011 over 500,000 accessions including original lines and their bulked or single-seed descendant were

available at the Nottingham Arabidopsis Stock Centre (NASC) at Nottingham University, UK (http://arabidopsis.info/). *A. thaliana* has been used as a model species in a diverse number of studies because of the large amount of information available on its physiology, development and molecular biology (Meyerowitz 1989). The entire life cycle, including seed germination, formation of a rosette plant, bolting of the main stem, flowering, and maturation of the first seeds, is completed in six weeks (Meinke *et al.* 1988). *A. thaliana* genome is organized into five chromosomes containing an estimated 20,000 genes and its genome was sequenced in 2000 (Initiative 2000). The genetic diversity of the specie is considerable and a diversity of individual mutations with known background lines is commonly available for study (Meyerowitz & Pruitt 1985; Meyerowitz 1989; Koornneef, Alonso-Blanco & Vreugdenhil 2004)

1. 2. Life history of *Arabidopsis thaliana*

1. 2. 1. Introduction

The life history of a plant can be defined as the suite characters that define the timing of crucial events in the life cycle of the species, which in plants include germination, the period of vegetative growth, the reproductive period and subsequently senescence (Pigliucci 2002). Features of individual life history characteristics are as follows.

1. 2. 2. Fertilization

It is commonly assumed that *Arabidopsis thaliana* is a nearly completely self-fertilizing species with an outcrossing rate estimated at ~1% (Shimizu *et al.* 2004), which has been an important reason for choosing this species as a model for genetics and molecular biology (Meyerowitz & Pruitt 1985; Meyerowitz 1989). This assumption has been made due to *A. thaliana* flower morphology, typical for inbreeding plants: small flowers, lack of scent and anthers situated close to the stigmata (Charlesworth & Vekemans 2005). The selfing rate in natural populations has been found to be very high (Charlesworth & Vekemans 2005) but as early as 1971, M. E. Jones (Jones 1971a) observed outbreeding in natural populations of *A. thaliana*. More recently, genetic variation found on local populations suggests regular gene flow between populations (Nordborg *et al.* 2005; Bakker *et al.* 2006). However, *A. thaliana* outcrossing is rarely observed under laboratory settings (Shindo, Bernasconi & Hardtke 2007) probably due to the absence of insects that have been suggested as pollen vectors (Hoffmann *et al.* 2003).

1. 2. 3. Seed, dormancy and germination

A single *Arabidopsis thaliana* plant under optimal conditions can produce more than 20,000 seeds by the end of its reproductive cycle (Meinke 1994). Seeds are very small size, typically only 0.5 mm long at maturity and with a dry weight about 20-30 μg. As with many other crucifers, *A. thaliana* seeds are produced in fruits known as siliques. Each silique contains two carpels and a central septum that separates two long rows of seed. Each seed is attached to the central septum through a funiculus that guides the entry of a pollen tube during fertilization and provides nutrients from the maternal plant

during embryo development. A typical silique grown under optimal conditions contains 40-60 seeds (Meinke 1994).

Reproductive development from fertilization to seed desiccation is usually completed in two weeks (at 23°C in 16 hour/8 hour light/dark cycles; Meinke 1994). As with other crucifers, siliques are arranged in a developmental progression along the length of the stem presenting young siliques at the tip of each stem, whereas, old siliques are located at the base. Plants grown under optimal conditions can produce as many as 500-600 siliques. The length of individual siliques varies with both developmental age and number of seeds (Meinke 1994).

Seed development in *A. thaliana* includes two major phases: embryo development and seed maturation (Raz, Bergervoet & Koornneef 2001). Embryogenesis starts with a morphogenesis phase and ends at the heart stage when all embryo structures have been developed (Mayer *et al.* 1991). This is followed by a growth phase during which the embryo fills the seed sac (Goldber, Paiva & Yadegari 1994). When the embryo growth phase concludes, cell division in the embryo stops. Latter during development, the seed, which contains a full size embryo, undergoes maturation during which food reserves accumulates and dormancy and desiccation tolerance develops (Goldber, Paiva & Yadegari 1994).

In *A. thaliana*, removal of the seed coat allows germination of non-germinating and strongly dormant genotypes, and dormancy in this species is described as coat-enhanced dormancy (Bewley 1997). However, the growth potential of the embryo also has an important role in dormancy processes (Bentsink & Koornneef 2002). This is controlled by several environmental factors such as light, temperature and the duration of seed storage after ripening (Koornneef, Bentsink & Hilhorst 2002).

There is a second type of seed dormancy due to embryo dormancy. Embryo dormancy is thought to be due to the presence of inhibitors, especially abscisic acid (ABA), as well as the presence of growth promoters like the gibberelic acid (GA). The loss of embryo dormancy is associated with a decrease in the ratio of ABA to GA (Taiz & Zeiger 2006). Normally, ABA accumulation in developing seeds is low during the early stages, reaching its peak during development when reserves are being synthesized, and declines as seed completes maturation. Absence of germination during seed

development is due to the external environment, the ABA content of the seed or both (Karssen *et al.* 1983; Berry & Bewley 1992).

For an annual plant species such as *A. thaliana* to persist in a given environment, seed germination must occur at a time of the year when environmental conditions are favourable for optimum establishment. Favourable conditions for germination and successful completion of the life cycle are specific to different ecotypes in *A. thaliana*. In some ecotypes, seeds are shed at a time when conditions are favourable for germination and remain optimal for completion of the life cycle. In these ecotypes seeds may lack dormancy and germinate during the season in which they are dispersed in order to obtain as many generations as it is possible. In other ecotypes seeds are dispersed at a time when environmental conditions are unsuitable for germination or when conditions are unsuitable at a given time of the cycle to complete it successfully. Timing of seed germination is critical for the performance and survival of the plant. The conditions that the plant encounters with germination are the same that the young seedlings must face. Germination timing strongly influences seedling survivorship (Cook 1980; Gross & Smith 1991; Masuda & Washitani 1992), plant fitness (Masuda & Washitani 1992; Donohue 2002), life history (Galloway 2001; Donohue 2002; Gallaway 2002), as well as selection on post-germination characters (Donohue 2002). Characterization on how germination timing influences fitness, phenotypic expression, and natural selection on life-history has provided valuable information on the pattern in which plants might be affected by environmental and evolutionary changes in their germination behaviour (Donohue 2002), and this is to have a major importance in competitive processes. The rate of evolution of germination timing may strongly influence the rate at which *A. thaliana* can expand its range and adapt to new environments (Donohue *et al.* 2005).

The mechanism of summer annual *vs.* winter annual life histories in *A. thaliana* is a function of both seed dormancy and vernalization requirements for flowering (Nordborg & Bergelson 1999). Investigating the relationship between the season of germination and subsequent life-history characters is relevant for understanding plant-plant interactions of summer and winter annual strategies. Standard ecological categorization of plant germination differentiates an annual such as *A. thaliana* into

"summer annual" accessions from "winter annuals". *A. thaliana* exhibit both summer and winter annual germination behaviour depending on the accession (Nordborg & Bergelson 1999). It should be noted that most summer-annual accessions can also follow a winter annual strategy of going through winter season as rosettes if conditions are adverse (Pigliucci 2003).

1. 2. 4. Vegetative development

Arabidopsis thaliana, as most dicotyledonous species, exhibits heteroblasty, which is a term referring to the age-dependent changes in the morphology of leaves (Goebel 1900). It is not clear what is the evolutionary role of heteroblasty in plants (Gould 1993; Gamage & Jesson 2007); but in *A. thaliana*, its leaves change in shape from the juvenile, early adult to the late adult phase (Telfer, Bollman & Poethig 1997). Between these two phases there is a brief transition phase, which is marked by the production of serrated leaves and an increase in the number of hyathode and trichome production on both surfaces (Martinez-Zapater *et al.* 1994; Telfer, Bollman & Poethig 1997; Tsukaya *et al.* 2000; Berardini *et al.* 2001). Rosette leaves during the vegetative phase change shape in correspondence with their positions from the early juvenile phase to the adult phase. This change in shape includes the shift of the widest region of the leaf from the base of the leaf towards the tip (Tsuge, Tsukaya & Uchimiya 1996). Later rosette leaves are larger, more elliptical, and more serrated (Telfer, Bollman & Poethig 1997). The first two rosette leaves are morphologically similar to cotyledons and share with cotyledons a reduced potential for trichome production (Poethig 1997; Telfer, Bollman & Poethig 1997).

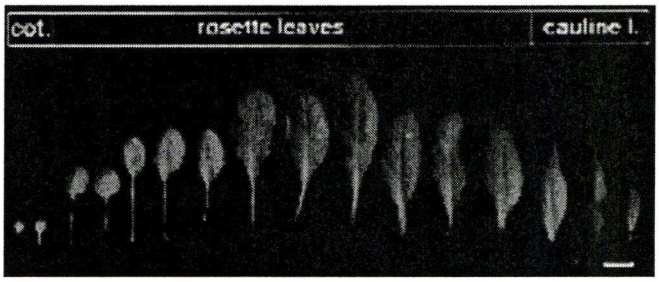

Figure 1. 1. Heteroblasty in *A. thaliana* (Columbia ecotype). The photograph shows gradual changes in the shape of leaves. From left: two cotyledons; eleven rosette leaves arrange in order of appearance; and three cauline leaves. Bar = 5 mm. See text for details (From Tsukaya, Shoda et al. 2000; presented with permission from the author).

1. 2. 5. Venation

Venation in *Arabidopsis thaliana*'s expanded vegetative leaves is brochidodromous, so it presents a primary midvein and a series of secondary veins shifting towards the edge of the leaf blade creating a series of loops formed by secondary veins connected by other secondary veins. The two secondary veins are placed closer to the base of the petiole of the leaf branch off the distal regions and join the rest of the leaf vascular system. The midvein reaches its maximum width at the basal region of the leaf and gradually diminishes in size distally (Candela, Martínez-Laborda & Luis Micol 1999). In *A. thaliana*, almost all veins are associated with the mesophyll (Haritatos, Medville & Turgeon 2000).

Leaf venation is required for the import and distribution of water and solutes to the leaf and export of photoassimilates to other regions of the plant. Vein density in leaves is an important factor determining its transport capacity: the higher the venation density, the more channels per area are available for conduction. Vein positioning limits photosynthesis via its influence on leaf hydraulic efficiency (Brodribb, Feild & Jordan 2007)

For a given transpiration rate, leaf water potential becomes less negative as vein density increases. In dicots such as *A. thaliana*, the main flow of water enters the leaf blade through the primary midvein, distributing the water to the secondary veins which distributes it laterally. The hydraulic pressure and flow velocity values are more negative as we approach the tip of the blade, and with decreasing order of the vein (Jeje 1985). The different hierarchical orders of the leaf veins are coupled to certain tasks of the

water supply. Lower order veins provide for fast, long-distance transport while local dispersion is carried out by higher order veins (Roth-Nebelsick *et al.* 2001).

1. 2. 6. Trichomes

Trichomes are the hairs that are present on the surface of the leaves and stems of many plants (Esau 1965). In *A. thaliana* and many other species, trichomes are the first cells that terminally differentiate on young leaf primordia (Larkin *et al.* 1996). *Arabidopsis* trichomes are highly specialized single cells that are expanded out of the plane of the epidermis. On leaves, these cells have an unusual branched shape consisting of a stalk and two to four branches (Larkin *et al.* 1997). Trichomes begin to form on the adaxial surfaces of leaves very early in leaf development, at a time when the developing epidermis is still dividing rapidly. In the Columbia ecotype, the average number of trichomes on fully expanded first leaves is 30.5 (± 0.9) (Larkin *et al.* 1996). Leaves produced early in rosette development lack trichomes on their abaxial surface, while adult leaves have trichomes on both surfaces. The production of abaxial trichomes appears to be regulated by the age, rather than the size of the plant and is promoted by gibberellins (Telfer, Bollman & Poethig 1997).

Leaf hair density is a complex character composed of two separable traits: leaf area and the number of hairs initiated per leaf. Leaf size is primarily controlled by the growth environment, whereas leaf hair initiation shows more genetically based variation (Roy, Stanton & Eppley 1999). Water limitation promotes leaves with a lower size and higher trichome densities are commonly found in dry environments (Ehleringer 1984)

Trichomes affect leaf absorption and boundary layer characteristics. A different leaf absorptance affects photosynthetical rates and leaf temperature. Indirectly, a change in the temperature of the leaf affects the rates of stomatal conductance and photosynthetic rates. Changes in the boundary layer affects directly photosynthesis and stomatal conductance by affecting the diffusion of gas exchange properties. The boundary layer itself directly affects leaf temperature by changing the rate of heat transfer from the leaf and indirectly by affecting photosynthesis and stomatal conductance (Ehleringer 1980). Trichomes have an adaptative value in arid environments allowing the leaf increased photosynthetic rates in the absence of hairs.

Also, trichomes reduce the effect of high lethal temperatures and reduce transpirational water loss (Ehleringer 1980).

1. 2. 7. Flowering

The switch from vegetative development to flowering is a crucial developmental transition in the life cycle of plants (Simpson & Dean 2002b). In *A. thaliana* the transition to the reproductive phase is associated with an enlargement of the apical meristem and initiation of flower meristems instead of leaf primordia on the sides of the apical meristem (Taiz & Zeiger 2006). The switch from vegetative to reproductive growth is apparent with the appearance of the main stem, rapidly bolting from the rosette. This elongated stem presents a series of cauline leaves below the first flower. The cauline leaves lack petioles (Tsukaya 1995; Tsuge, Tsukaya & Uchimiya 1996). Inflorescences, which are open racemes with typical crucifer flowers, subsequently also develop from axillary buds in some of the rosette leaves and in most cauline leaves.

At a given point in their life cycle, annual plants such as *A. thaliana* undergo a developmental transition from vegetative to reproductive development. Physiological and genetic analysis of flowering has shown that multiple environmental and endogenous inputs influence the timing of this switch. Thus, two different pathways have been defined that genetically control the switch from vegetative to reproductive growth: the autonomous and the promoting pathway. The autonomous pathway is not influenced by some factors included in the promoting pathways such as light requirements and endogenous levels of gibberellins (Boss *et al.* 2004).

A. thaliana, like any other plant senses the changes in day-lengths associated with seasonal changes though the leaves, where a long distance signal called florigen is transmitted through the phloem although the identity of this signal remained unclear for many years. In the last few years, it was believed that this "florigen signal" was messenger RNA that travelled from *the FLOWERING LOCUS T* (*FT*) in plant leaves to the shoot apical meristem (SAM) (Huang *et al.* 2005) but recent studies showed how this mobile signal inducing flowering in *A. thaliana* is the FT protein itself rather than mRNA (Corbesier *et al.* 2007).

Regulation factors that regulate flowering in *A. thaliana* can be classified as promotion, enabling and resetting pathways (Boss *et al.* 2004). The promoting factors are photoperiod, gibberellins, temperature, and light conditions. Enabling pathways determine the activity of repressors of flowering, which we explain in a following section on vernalization processes. Finally, resetting pathways are required to reset the expression states of floral genes during formation of the gametes or during embryo development.

A. thaliana plants pass through a juvenile phase, in which flowering does not occur, to ensure enough reserves to sustain flower development but, after that phase inhibition of the time of flowering depends on environmental cues. Time of flowering, is also known to vary within and among different populations of *A. thaliana* (Westerman 1970b; Westerman 1970a; Westerman 1970c; Westerman & Lawrence 1970; Jones 1971a; Jones 1971c; Jones 1971b). Late flowering is associated with greater vegetative size: height, number of leaves and rosette diameter (Westerman 1970a; Jones 1971c) which has been suggested to relate to an increased reproductive ability (Aarssen & Clauss 1992; Clauss & Aarssen 1994). Although in general *A. thaliana* is regarded as a long day plant, its flowering critical value is low (Ratcliffe 1961). In general, longer days and vernalization promote earlier flowering.

In winter annual ecotypes flowering is accelerated by conditions that indicate the transition from winter and the onset of spring and summer such as a long period of low temperatures.

Vernalization constitutes a period of cold temperature (1 to 3 months of ~1° to 10°C depending on species or populations) (Simpson & Dean 2002b; Simpson & Dean 2002a). Vernalization is a reproductive strategy to ensure they over winter as a rosette. Molecular studies of vernalization processes in *A. thaliana* started with the work of Klauss Napp-Zinn in the sixties (Napp-Zinn 1961; Napp-Zinn 1962; Napp-Zinn 1969; Napp-Zinn 1985) who described a monogenic trait with dominant alleles of *FRIGIDA* (*FRI*) conferring a vernalization requirement. Rapid cycling ecotypes such as Columbia, and Landsberg erecta, which are not winter annuals and therefore do not need vernalization to trigger an earlier flowering, present recessive alleles for *FRI* (Johanson *et al.* 2000). In recent years genetic pathways of the vernalization process have been

defined by which *FRI* encodes for a protein that promotes the accumulation of *FLOWERING LOCUS C* (*FLC*) which is directly involved in the repression of flowering through the control the response to vernalization (Sheldon *et al.* 2000; Michaels & Amasino 2001). It has been suggested that early flowering is a response to uncertain environments characterized by short seasons while late flowering would be advantageous when the environment is more predictable and presents longer seasons (Westerman & Lawrence 1970; Jones 1971c; Pigliucci & Schlichting 1998). Early flowering ecotypes of *A. thaliana* tend to produce more yield and be fast-growing, being more successful under stressful conditions (Pigliucci & Schlichting 1998) such as in high density stands than late flowering ecotypes that germinate before exposure to winter and low temperatures in the vegetative state.

Variation in flowering time represents a complex set of trade-offs. Time of bolting is genetically correlated with the number of rosette leaves. Genes conferring early flowering can decrease the productivity of *A. thaliana*, producing less leaves and producing seed earlier but at the cost of a lower reproductive fitness (Mitchell-Olds 1996). Under density induced but under stressful conditions such as that associated with plant competition for a limited resource, early flowering may become an advantage.

1. 2. 8. Senescence

A. thaliana is a monocarpic species, and monocarpic plants have a very characteristic senescence process that occurs once the plant has reached the end of the reproductive phase. Thus, cessation of growth in the shoot and senescence are separate processes, therefore, anything that postpones reproduction in monocarpic plants will delay the death of the plant (Nooden 1984). However, this pattern does not occur in all monocarpic species, and the *Brassicaceae* which includes *A. thaliana*, are an exception within monocarpic plants and senescence may not be controlled by the reproductive stage; so senescence is not linked with the end of the reproductive phase (Hensel *et al.* 1993; Nooden, Hillsberg & Schneider 1996; Nooden & Penney 2001).

The most remarkable change in leaf senescence is associated with chlorophyll degradation and a decline in photosynthetic capability (Matile, Hortensteiner & Thomas 1999). Chlorophyll catabolism initially occurs at a basal turnover level, but is increased

during senescence and fruit ripening (Matile, Hortensteiner & Thomas 1999; Jiang *et al.* 2007). When *A. thaliana* plants age from 30 to 45 d, net photosynthesis declines by 40%. Such a decline is entirely caused by increased diffusion limitations to CO_2 transfer, of which decreased mesophyll conductance is the largest. Age-induced photosynthesis decline in *A. thaliana* is initiated by decreased mesophyll conductance and not by chlorophyll degradation and protein breakdown (Flexas *et al.* 2007a; Flexas *et al.* 2007b).

1. 3. Research aims

As described previously, the diversity of genotypes available for study with *A. thaliana*, offers the opportunity to investigate the role of individual genes on physiological and growth characteristics by comparative analysis of the performance of individual mutants. Linking photosynthetic performance to growth and subsequently life history characteristics and competitive interactions amongst genotypes represents a major challenge to understanding ecological adaptation within species. The primary objective of the research work reported in this thesis was to investigate photosynthetic differences amongst *A. thaliana* mutants and the consequences of these differences to growth, flowering and competitive interactions. This was achieved by a critical experimental examination of a stomatal density mutant of *A. thaliana* and its wild-type.

1. 4. Overview of stomatal densities in plants and *Arabidopsis thaliana* stomatal density mutants

Leaves possess a waxy cuticle with low permeability to water that is an effective protection against desiccation. It has been estimated that only ~5% of water lost by leaves is cuticular. In order to regulate the entry of gaseous CO_2 for photosynthesis, plants present stomatal pores linking intercellular spaces to the atmosphere, a physiological characteristic that has major implications for ecological processes. Stomata played an important role in plant evolution allowing them to move from water to land letting gas exchange and limiting water loss. The stoma is an epidermal structure that is formed by two guard cells surrounding a pore whose width is regulated. Guard cells in *A. thaliana* as in other dicots are kidney-shaped cells that have the ability of modifying their shape with alterations in cell turgor thus affecting pore width (Nadeau & Sack 2002). The key factor determining stomatal resistance is the width of this pore in between the guard cells. Stomatal diffusion resistance increases exponentially with the reduction in pore width. Stomatal conductance therefore is directly proportional to pore width (Larcher 1995), Stomatal pores link intercellular spaces to the atmosphere, a physiological characteristic that has major implications for ecological processes. This continuity allows carbon dioxide to reach the plant's mesophyll chloroplast for photosynthetic fixation (Taiz & Zeiger 2006).

Stomatal aperture is a compromise between conservation of water and optimisation of CO_2 fixation (Willmer & Fricker 1996). Plants cannot take up the necessary atmospheric CO_2 for their photosynthetic activity without simultaneously allowing outward diffusion of water through the stomatal pores. This is even more problematic due to the fact that the diffusion rate of water vapour is 1.6 times greater than CO_2 (McPherson & Slatyer 1973). Stomatal closure can serve as a rapid and effective drought-avoidance response, however, prolonged stomatal closure is not sustainable as stomatal CO_2 uptake is reduced and will limit photosynthetic assimilation and growth (Farquhar & Sharkey 1982; Schulze 1986; Valladares & Pearcy 1997; Juenger *et al.* 2005).

Stomatal density plays an important role on the water use efficiency of higher plants (Woodward 1987; Woodward & Bazzaz 1988; Mansfield, Hetherington &

Atkinson 1990; Woodward 1993; Woodward & Kelly 1995; Woodward, Lake & Quick 2002) affecting the response of plants to drought conditions (El-Sharkawy, Cock & Hernandez 1985). In the absence of variations in stomatal size, stomatal density determines the maximum stomatal conductance g_s that a leaf presents per unit area (Drake, Gonzalez-Meler & Long 1997b). Stomatal densities are species specific, although they vary with environmental conditions in order to adust gas and water relations to an optimum (Larcher 1995; Bergmann 2004).

Several studies have explored how stomatal densities in relation with climate conditions (Woodward 1987; Woodward & Bazzaz 1988; Woodward 1993; Woodward & Kelly 1995; Beerling & Woodward 1997; Woodward, Lake & Quick 2002; Hetherington & Woodward 2003) in order to improve water use efficiency. Also the relationship between stomatal densities, photosynthesis and growth have been extensively reviewed in crop species with the objective of improving yield by improving water use efficiency (Heichel 1971; Miskin, Rasmusson & Moss 1972; Yoshida , Dale & Rasmusson 1975; Heursel, Ceulemans & Ibrahim 1987; Jones 1987; Lu & Zeiger 1994; Radin *et al.* 1994; Percy *et al.* 1996; Kundu & Tigerstedt 1999; Yu 2001; Liao, Chang & Wang 2005; Yousufzai, Siddiqui & Soomro 2009). This relation has been also studied in invasive species (Walton 1974). These studies compare different species or cultivars, making comparisons involving different phenologies or genetic background that may complicate the interpretation of the results.

Stomatal number varies in different organs of *A. thaliana* and are present in the mature epidermis of the different aerial organs of the plant excepting petals and stamen filaments Number, distribution, size, shape and mobility of stomata are species specific, although they vary with environmental conditions (Larcher 1995; Bergmann 2004).

Stomata normally follows several patterning rules: first, they are formed though a series of asymmetric divisions and secondly, they are patterned so never two stomatal complexes are adjacent to each other and thirdly, stomatal density is controlled by environmental conditions (Bergmann 2004). The epidermis of germinating *A. thaliana* seedlings does not present cellular differentiation. But within 24 hours stomatal development cellular divisions start to take place creating meristemoid cell (Bergmann 2004). An unequal division of a meristemoid cells results in the formation of (1) smaller

cell which are going to be precursors for the stomatal guard cell and (2) a larger cell that is going to work as a "pavement" cell and is not going to originate a stomatal cell unless it would divide unequally (Geisler, Nadeau & Sack 2000). Meristemoids can divide again asymmetrically up to three times, each time retaining the meristemoid character in the smaller daughter cell from the division (Geisler, Nadeau & Sack 2000). Finally, the meristemoid cell differentiates into a guard mother cell (GMC) which divides a single time, symmetrically, to form the two paired guard cells of the stoma (Zhao & Sack 1999).

There are mutations in four genes in *A. thaliana* that result in altered stomatal densities, stomatal clusters and also presenting single stomata. These four genes are *YODA* (YDA), *TOO MANY MOUTHS* (TMM), *FOUR LIPS* (FLP), and *STOMATAL DENSITY AND DISTRIBUTION* (SDD1) (Yang & Sack 1995; Berger & Altmann 2000; Lukowitz *et al.* 2004).

1. 5. 1. *Tmm1-1* mutant (*col-gl1* background)

The *too many mouths* (*tmm*1-1) mutant in *A. thaliana* presents a phenotype with stomatal clustering (Yang & Sack 1995). TMM encodes a leucine-rich repeat (LRR) containing receptor-like protein expressed in proliferative post-protodermal cells (Nadeau & Sack 2002). *tmm1-1* is a recessive mutation in the TMM gene which is required for cells to respond to their position during stomatal development and participates in intercellular signalling (Nadeau & Sack 2002). Also, it seems that *tmm* eliminates stomata in several *A. thaliana* organs (Geisler, Yang & Sack 1998)

1. 5. 2. *Flp* mutant (*columbia-gl1* background)

The *four lips* mutation is characterized by a phenotype in which there is a presence of two adjacent stomata and a number of unpaired guard cells (Yang & Sack 1995). The frequency of these stomatal clusters seems to vary among the different epidermal tissues (Geisler, Yang & Sack 1998) Stomatal clusters of *flp* seem to be distributed in a similar pattern to the wild-type, with each cluster positionally equivalent to a single stoma in wild-type (Yang & Sack 1995). Like *TMM*, the *FLP* gene seems to

prevent clustering (Yang & Sack 1995) but does not seem responsible of meristemoid initiation and seems to act downstream from *TMM* (Geisler, Yang & Sack 1998).

1. 5. 3. *Yda1* and *yda2* mutants (*ler-0* background)

YODA (*YDA*) is a mitogen-activated protein kinase kinase (MAPKK) gene (Bergmann, Lukowitz & Somerville 2004). Loss of function mutation in the *YDA* gene results in too many cells differentiating into guard cells, and activation of *YDA* produces plants that completely lack guard cells. The *yda1* and *yda2* mutations in the *YDA* gene (Bergmann, Lukowitz & Somerville 2004) results in plants with a very distinct phenotype in which almost all the cells at the plant surface are guard cells. This overproduction of stomata makes many of the seedlings die and those surviving produce small adult phenotypes with sterile flowers.

1. 5. 4. *Sdd1-1* and *sdd1-2* mutants (Columbia *C24* and *col-5* background)

The *stomatal density and distribution* (*sdd1-1* and *sdd1-2*) mutants (Yang & Sack 1995), are a point mutation in a single gene (*SDD1*) which is predicted to produce subtilisin-like protease that is probably secreted mediating developmental processes during stomatal development as described above. It presents a formation of extra adjacent stomata and an increase of 2.5 fold in stomatal density in rosette leaves, but the internal leaf architecture or other apparent features in the morphology of leaves does not differ from wild-type (Berger & Altmann 2000). The *SDD1* gene is believed to act as a processing protease involved in the mediation of a signal controlling the development that leads to the formation of guard cells (Berger & Altmann 2000).

The *sdd1-2* mutation has the accession *col-5* as its wild-type. The genotype *col-5* is the result of crossing the natural occurring *gl1-1* glabrous polymorphism into the *col-0* wild-type. *Col-5* lacks trichomes on stems and leaves. Seeds for *sdd1-2* and *col-5* were kindly donated by Dr. Fred Sack from the University of British Columbia.

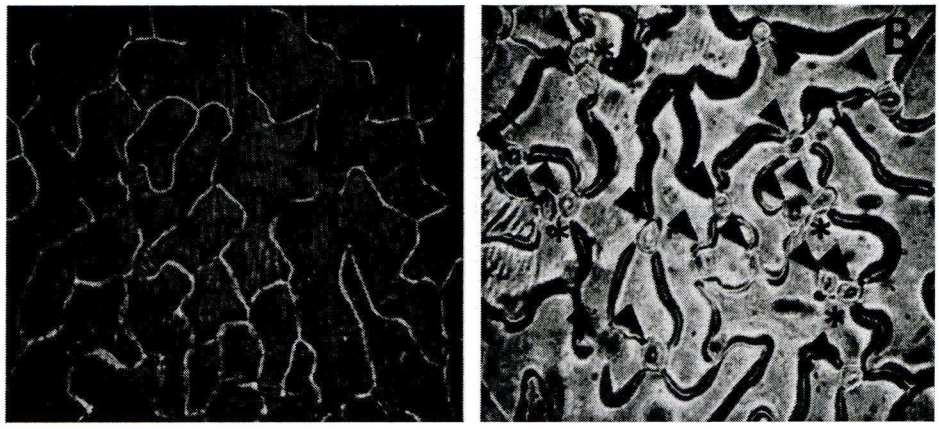

Figure 1. 2. A. Epidermal peeling of *col-5* and B. *sdd1-2*. Arrows point to stomatal units and asterices to paired stomatal units.

The *sdd1-2* mutant was chosen for study because it provided a phenotype with an increase in stomatal density and with probable consequences for CO_2 assimilation, stomatal conductance, water use efficiency, growth and competitive interactions.

1. 5. Chapter overview

Chapter 2 present the results of preliminary investigations into growth conditions for use in subsequent experimentation. The effect of different types of soils and soil volumes were examined for vegetative and reproductive biomass.

Work in Chapter 3 investigates the photochemistry of *sdd1-2* and *col-5*, its background line and the light responses of both genotypes. The motivation for this work was to assess any differences in photochemical attributes that may be a consequence of the mutation in the *sdd1-2* to assess the degree to which differences in growth and photosynthetic properties are solely due to differences related to different stomatal densities. The photosynthetic capacity of each genotype is considered.

Chapter 4 considers CO_2 assimilation, stomatal conductance, water use efficiency and photosynthesis in the two mutants. This chapter also explores growth and flowering characteristics of both genotypes in relation to water availability.

Chapter 5 examines competitive interactions in *A. thaliana* in response to density and water availability affecting biomass production. Intra-genotypic interactions in *col-0* are assessed by yield density responses; and complemented with a surface response

analysis comparing *sdd1-2* and *col-5* in partial intra- and inter-genotypic interactions under two water regimes.

Finally, Chapter 6 explores inter-and intra-genotypic competition in relation to water availability exploring both above- and belowground responses to competition.

Chapter 2. Growth of *Arabidopsis thaliana* in relation to soil type and soil volume.

2. 1. Introduction

Plants compete for several soil resources such as water and essential mineral nutrients, whereas aboveground competition primarily involves a single resource, light (Casper & Jackson 1997). Understanding of plant competition is often focused on aboveground studies (Cahill, Kembel & Gustafson 2005). However, belowground competition often reduces plant fitness more than aboveground competition (Wilson 1988b; Ferrero-Serrano *et al.* 2006; Ferrero-Serrano *et al.* 2008; Ferrero-Serrano, Hild & Mealor 2009). It remains important to attempt to address how belowground competition relatively affects aboveground and total plant performance. Competition for belowground resources can be intense in natural communities, often limiting plant growth and establishment (Casper & Jackson 1997).

Shoot: root ratios often decrease with limited nutrient availability and increase under low irradiance. Plasticity in shoot: root ratios is thought to reflect optimal allocation of plant biomass for resource uptake and allocation adjustments are presumed to maximize capture of the most limiting resources (Bloom, Chapin & Mooney 1985; Chapin *et al.* 1987). A large amount of empirical data generally supports the idea that, in the absence of plant competition, resource availability influences biomass allocation (Wilson 1988a).

A colonizing individual plant may not compete successfully for water and essential nutrients against the root system of already established plant individuals. This is particularly important in plant communities, where individuals have very high rooting densities that effectively exploit limited soil resources. As a result, there is a reduction in the physical volume within which resources are available for competition so-called "biological space" (Cornforth 1968; Ross & Harper 1972; McConnaughay & Bazzaz 1991). The belowground biological space of an individual plant can be regarded as the volume of soil from which resources such as water and essential mineral nutrients are acquired but this biological space has also been shown to be related to the individual's

position within the area as defined by its neighbours and also its time of emergence and stage of development relative to these neighbours.

The arrival of new colonizing individuals is limited by their establishment and persistence if physical underground space *per se* is unavailable for the normal deployment of roots. This availability physically affects root growth, and also governs water and nutrient levels which if limiting may lead to a reduction in whole plant growth and fitness. Many hydroponic studies have altered rooting volume while maintaining a continuous flow of aerated, nutrient-rich solution to roots. These studies document changes in root architecture and morphology that result in reduced water, and probably nutrient, acquisition and translocation to the shoot, reduced hormone production and translocation, or a combination of these factors (McConnaughay & Bazzaz 1991).

Studies on the release of belowground physical space as a result of gap formation within vegetation, were conducted (McConnaughay & Bazzaz 1991). These authors concluded that the physical underground space, released from gap formation, may influence the performance of colonizing annuals beyond providing access to other soil resources, such as nutrients and water.

This important ecological process by which the availability of belowground resources determines the growth of the plant, is of relevance to the research directions of this thesis since a principal aim of the work is to make critical comparisons of the growth and relative performance of genotypes of *A. thaliana*. The purpose of this studies reported in this Chapter was to investigate how the amount of belowground physical space available to a plant interacts with the availability of soil supplied resources that are essential for growth and competition. The approach taken was to examine the performance of *A. thaliana* grown in different types of soil compost in varying soil volumes. A subsidiary objective was to determine appropriate experimental soil growth regimes for subsequent experimentation.

2. 2. Materials and Methods

Three soil growth media, John Innes No 1, 2 and 3 composts were supplied in 10 x 10 x 11 cm (600 ml) square pots, filled to different extents to provide four different soil volumes 600, 450, 300 and 150 ml. As soil volume decreased from 600 ml, the remaining space at the bottom of the pot was filled with plastic beads keeping the same surface pot area for all treatments. This was important in order to secure the same for soil surface evapotranspiration with a standard watering regime common to all the pots. John Innes commercial soil compost is composed of a John Innes base fertiliser (N, P, K; 5-7.7-10) with various additions of peat based growing medium of negligible nutrient content. Compost No 1 has the lowest nutrient content and is generally used for sowing large seeded commercial plants and pricking out seedlings and rooted cuttings. Compost No 2 is used for potting-on most plants, and contains twice the added nutrients of No 1. Compost No 3 has the highest nutrient content and is used for growth of mature plants.

Performance of two ecotypes of *A. thaliana* was examined: Columbia (*col-0*) and Landsberg (*ler-0*) obtained from NASC stock center. Three seeds of each ecotype were sown per pot and seedlings thinned to a single individual as soon as germination was completed. Plants were grown in a growth chamber at 21°C with 160 μmol m^{-2} s^{-1}. The chamber was maintained at 21 °C during the day and 19 °C during the night with 50% relative humidity and light intensity in the range of 150 and 200 μmol m^{-2} s^{-1} PPFD. Plants were uniformly watered from the top as needed to maintain a moist soil. Destructive harvest took place 40 days after planting, with vegetative and reproductive biomass measured after oven drying at 60°C for 48 h and weighed.

The experimental design involved 24 different treatment combinations, 2 genotypes x three soil media (John Innes No 1, 2 and 3) x four soil volumes with five replications at the time of harvest (40 days after emergence) were taken. Statistical analysis was conducted using the statistic program package R (http://cran.r-project.org/). I conducted an ANOVA using Type III sum- of-squares. Five repetitions were used per treatment. Normality of the residuals was tested with the Shapiro–Wilk test. Homogenity of variance was tested using the Fligner-Killeen test. The post-hoc Tukey pair-wise comparisons of means was used at the $\alpha = 0.05$ level.

2. 3. Results

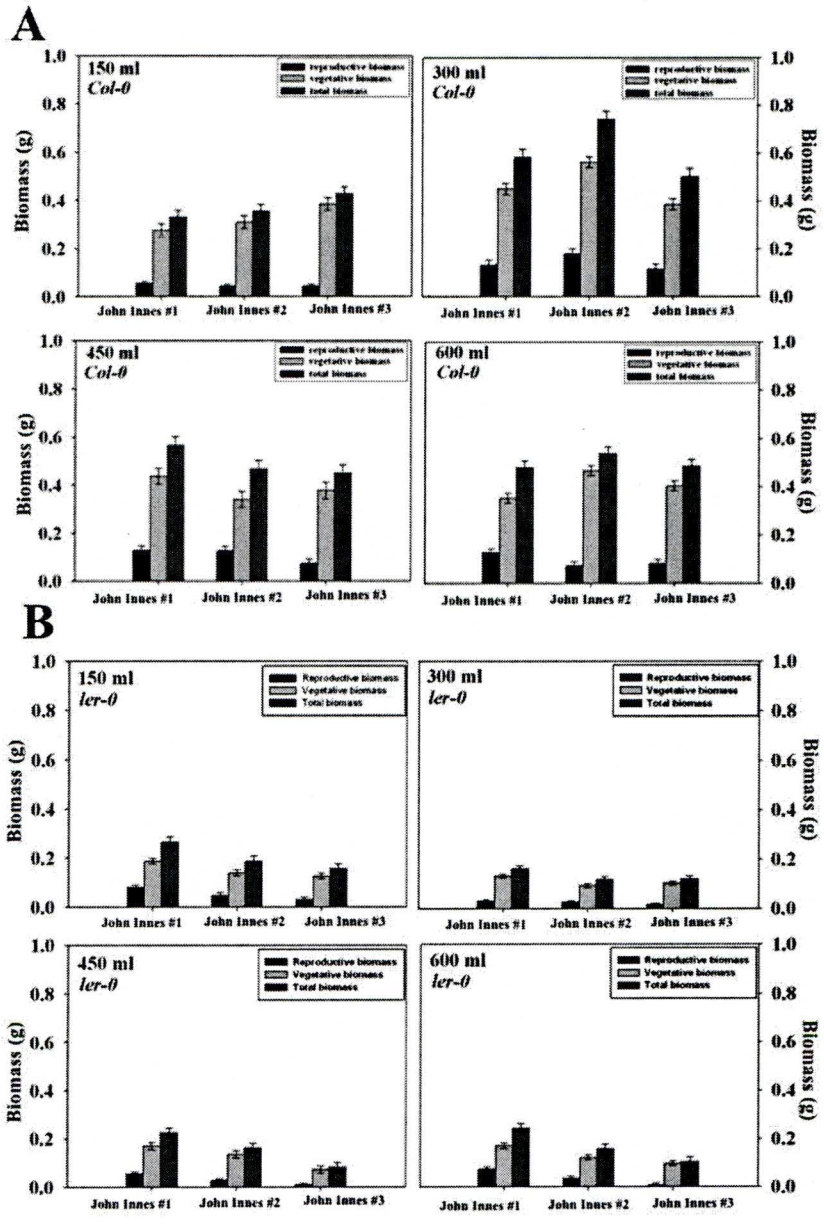

Figure 2. 1. Dry biomass (reproductive, vegetative and total biomass) production of A.) *A. thaliana* Columbia (*col-0*) and B.) Lansberg (*ler-0*) ecotypes in relation to media and soil volume. Results are the mean of five replicate plants ± SE.

Table 2. 1. Two-factor ANOVA table for vegetative, reproductive and total biomass for *col-0* in relation to soil volume and type of soil. For each comparison there were two factors: soil volume and soil type. Soil volume is a factor with four levels: 150, 300, 450 and 600 ml. Soil type has three levels: John Innes No 1, John Innes No 2 and John Innes No 3.

	Source of variation	df	SS	F value	P
Vegetative biomass	***Volume***	1	33485	2.884	0.096
	Soil type	2	1333	0.057	0.994
	Volume x soil type	2	9892	0.426	0.655
	Residuals	50	580527		
Reproductive biomass	***Volume***	1	7401	1.494	0.227
	Soil type	2	8482	0.856	0.431
	Volume x soil type	2	4746	0.479	0.622
	Residuals	50	247708		
Total biomass	***Volume***	1	72370	4.006	0.051
	Soil type	2	7948	0.22	0.803
	Volume x soil type	2	16317	0.452	0.639
	Residuals	50	903339		

Figure 2. 1 shows that there were significant differences between *col-0* and *ler-0* genotypes in terms of all three measures of growth. The *col-0* genoype produced larger (ca x 2.5) plants than the *ler-0* genotype. In the *col-0* ecotype (Figure 2. 1A) no significant differences were found in vegetative, reproductive or total biomass in relation to the available soil volume to determine growth or with the different types of compost used and neither did these factors interact to determine growth (Table 2. 1).

Table 2. 2. Two-factor ANOVA table for vegetative, reproductive and total biomass for *ler-0* in relation to soil volume and type of soil. For each comparison there were two factors: soil volume and soil type. Soil volume is a factor with four levels: 150, 300, 450 and 600 ml. Soil type has three levels: John Innes No 1, John Innes No 2 and John Innes No 3.

	Source of variation	df	SS	F value	P
Vegetative biomass	***Volume***	1	1770	1.206	0.277
	Soil type	2	44003	14.999	<0.01
	Volume x soil type	2	1593	0.543	0.584
	Residuals	53	77744		
Reproductive biomass	***Volume***	1	1001	1.384	0.245
	Soil type	2	15946	11.021	<0.01
	Volume x soil type	2	742	0.513	0.602
	Residuals	50	36171		
Total biomass	***Volume***	1	5694	1.843	0.18
	Soil type	2	121351	19.64	<0.01
	Volume x soil type	2	4162	0.674	0.514
	Residuals	50	163739		

However, in the *ler-0* accession (Figure 2. 1B), there was a significant decrease in vegetative, reproductive and total biomass as the nutrient content of the soil increased (John Innes No1 – No3). Graphically, this was particularly evident in the treatments with 150, 450 and 600 ml of soil (Figure 2. 1). No significant differences in biomass resulted from changing volume as found with *col-0*.

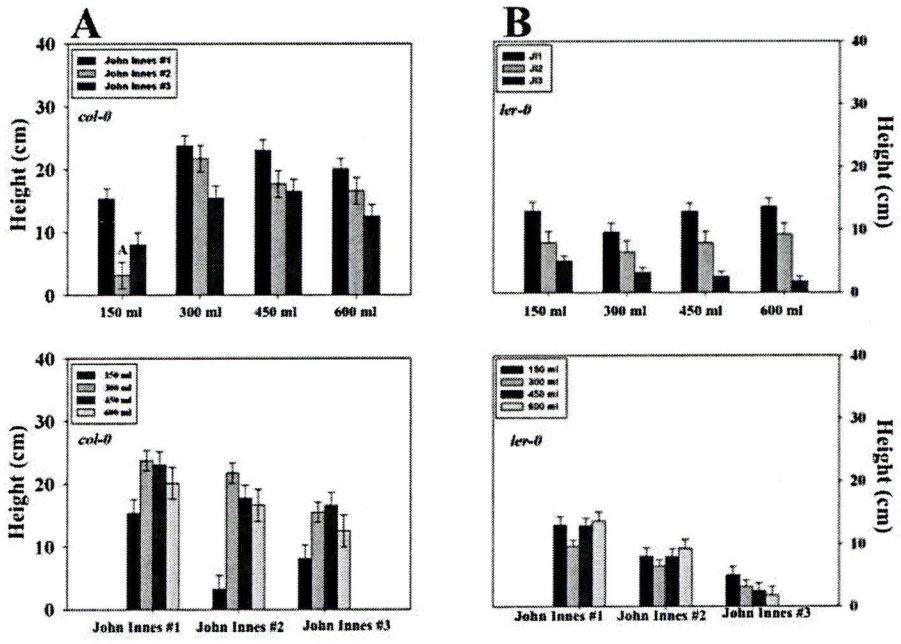

Figure 2. 2. Mean bolt heights of Columbia, *col-0,* and Langsberg, *ler-0*, ecotypes at 40 DAE at different soil volumes (150, 300, 450, 600 ml) in relation to compost type. Results are the mean of five replicate plants ± SE.

Table 2. 3. Two-factor ANOVA table for bolt height for *col-0* and *ler-0* in relation to soil volume and type of soil. For each comparison there were two factors: soil volume and soil type. Soil volume is a factor with four levels: 150, 300, 450 and 600 ml. Soil type has three levels: John Innes No 1, John Innes No 2 and John Innes No 3.

Pairwise comparison	Source of variation	df	SS	F value	P
col-0	*Volume*	1	390.79	6.311	<0.05
	Soil type	2	592	4.78	<0.05
	Volume x soil type	2	19.99	0.161	0.851
	Residuals	50	30961		
ler-0	*Volume*	1	0.26	0.028	0.866
	Soil type	2	733.73	40.52	<0.01
	Volume x soil type	2	38.81	2.143	0.128
	Residuals	51	461.75		

Both soil volume and type of compost had significant effects on bolt height after 40 DAE in *col-0* (Table 2. 3). With increasing nutrient status, bolt height declined, averaging over soil volumes greater than 150 ml. Similarly heights declined in a

consistent manner as soil volumes increased over 300 ml (Figure 2. 2). At 150 ml soil volume, the yield on John Innes No 2 was depressed in comparison to yields on the other two compost regimes. Bolt heights of *ler-0* were shorter than those observed in *col-0* and only influenced by compost type. Again, the higher volumes of more nutrient rich compost (600 ml, John Innes No 3) resulted in shorter bolts (Figure 2. 2B).

2. 4. Discussion

Restricting the root growth of individual plants in the absence of interaction with other plants may result in changes in both root architecture and morphology and therefore nutrient and water uptake by the plant, leading to a reduced growth and reproductive yield.

From the results obtained, we can observe how in the *col-0* accession there are no differences in vegetative, reproductive or total biomass when grown at different volumes of soil, so plants are not limited by the studied soil volumes when growing alone. However, in the *ler-0* ecotype, a tendency to a decrease in vegetative, reproductive and total biomass is observed as nutrient content of the soil increases (John Innes #3). This may suggest that one individual of this ecotype growing by itself, finds the optimal quantity of nutrients on John Innes #1 media. Media did not alter growth on *col-0*.

In the growth conditions of this experiment, the volume of soil presented for growth of both genotypes did not influence the performance of plants in either vegetative or reproductive biomass; with the exception of the lowest volume of 150 ml for *col-0*. This is an indication for future experimentation that resource limitation was not dependent on soil volume. Biomass production was as expected, increased by higher nutrient levels. On the other hand bolt height at 40 DAE, which is a proxy for flowering and seed production decreased with nutrient availability. Higher nutrient levels are likely to promoted continued vegetative growth and as a result there is a delay in the switch to the reproductive stage. However nutrient limitation has been reported to delay flowering for both *col-0* and ler-0 (Nord & Lynch 2008). This may be due to the fact that plants under nutrient limitation present reduced RGR's compared to those growing under

optimal conditions. Thus, they will take longer to attain the minimum or "critical" size that plants have been proposed to reach necessarily to be able to flower (Werner 1975).

Meyre, Leonardi et al. (2001) studied the different growth strategies that *col-0* and *ler-0* presented when subjected to water stress. *Ler-0* accelerated flowering producing higher reproductive biomass, and was argued to present and 'escape' strategy when subjected to water limitation. In contrast, *col-0* followed a 'tolerance' strategy with a later switch to the reproductive stage and a larger rosette biomass; with lower aboveground to belowground ratios, water use efficiency (WUE), relative water content (RWC) and later leaf senescence (Meyre *et al.* 2001).

This study suggested that *col-0* was less responsive to soil media and different volumes than *ler-0*. The fact that *ler-0* was more sensitive to those suggest that under these conditions, this ecotype exhibited this contrasting "escape" strategy. Also, the lower shoot to root biomass reported for *col-0* (Nord & Lynch 2008) suggest that, even though this ecotype is more capable of belowground growth than *ler-0* variation in soil conditions (type and volume) did not have a significant effect on growth.

This concludes that *A. thaliana*, and particularly the *col-0* ecotype, which is the background line for the mutants studied in this Thesis is not particularly sensitive to changes in the available soil space when growing alone. This information was useful to determine the appropriate soil media and type of soil as well as to discard any limitation of pot size on the development of plants growing alone.

Chapter 3. Photochemistry and photosynthetic light response of the *Arabidopsis thaliana* mutant, *sdd1-2*

3. 1. Introduction

The *SDD1* gene encodes a subtilin-like protease involved in the mediation of a signal controlling the epidermal cell development that leads to the formation of guard cells (Yang & Sack 1995; Berger & Altmann 2000). The *sdd1-2* mutant in *Arabidopsis thaliana* (*col-5* background) is typified by the formation of supplementary, adjacent stomatal guard cell pairs and a 2.5-fold increase in stomatal density in rosette leaves. The stomatal density of *sdd1* mutants varies within the leaf, being doubled in the adaxial epidermis and increased three to four fold in the abaxial surface (Berger & Altmann 2000; Von Groll, Berger & Altmann 2002). However, the internal leaf architecture and other apparent features of the morphology of the *sdd1* leaves do not differ from wild-type (Berger & Altmann 2000).

Previous work on a second mutant allele of the *SDD1* gene (*sdd1-1*), showed that in plants pre-adapted to high light, increased stomatal density enabled higher CO_2 assimilation rates than in the wild-type (Schlüter *et al.* 2003). As argued in Chapter 1, changes in fitness that could accrue from the presence of increased stomatal density might be attributed to increased efficiency of the photosynthetic process *per se,* or increased CO_2 assimilation as a consequence of a higher rate of leaf-atmosphere gas exchange. The first objective of the work reported in this Chapter was to explore whether the *sdd1-2* mutant had perturbations of its photosynthetic capacity, through the study of the chlorophyll fluorescence properties of the mutant compared to its wild-type.

Chlorophyll fluorescence is a key experimental procedure for the assessment of plant photochemistry. It enables detailed characterisation of the efficiency of Photosystem II (PSII) in using the energy absorbed by chlorophyll. This is of significance because quantification of electron flow through PSII is a valuable proxy for the photosynthetic capacity of PSII (Genty, Briantais & Baker 1989; Edwards & Baker 1993; Maxwell & Johnson 2000b). Chlorophyll fluorescence can be used to determine

whether or not the mutation has resulted in the inhibition of, or damage to, electron transfer from PSII (Bolhar-Nordenkampf *et al.* 1989).

When light energy is absorbed by chlorophyll it attains an excited state. Excited chlorophyll molecules return to their ground state by following the photosynthetic pathway and by dissipation of excess in energy either as heat or fluorescence (Demmig-Adams & Adams III 1992). These three processes compete such that an increase in energy following one pathway will result in less energy following the remaining two. Therefore, quantifying the yield of chlorophyll fluorescence will give information about changes in the efficiency of photochemistry and heat dissipation. When compared to the total amount of light absorbed, chlorophyll fluorescence is very small only representing 1 to 2% of the total amount of light absorbed by the leaf (Maxwell & Johnson 2000b). However, fluorescence is very easy to measure because during this process, the wavelength of fluorescence is slightly longer (and with lower energy) than the wavelength of absorption and thus very reliable to assess the efficiency of the photochemistry and heat dissipation characteristics of plants (Maxwell & Johnson 2000a).

In practice chlorophyll fluorescence is assessed by 'switching off' non-photochemical and photochemical quenching. The photochemical characteristics occurring in leaves in steady state conditions pre-adapted to the dark, in the absence of incident radiation are measured first and then followed by measurement in the presence of radiation. Under dark-adapted conditions, the cooperation of PSI and PSII photo systems is impaired due to the inactivation of key enzymes in the ribulose-1-5 biphosphate (RubP) cycle (Vu, Allen Jr & Bowes 1984), the depletion of intermediate metabolites (Vu, Allen Jr & Bowes 1984) and the inhibition of excitation-energy transfer in PSI (Canaani & Malkin 1984). The minimal level of fluorescence (F_o) is calculated under dark-adapted conditions before exposure to pulsed lighting (Figure 3. 1).

The leaf is then subjected to a short saturating intensity light pulse (0.8 *s* pulse of 10000 μmol m^{-2} s^{-1} PAR). This has the effect of momentarily closing all PSII reaction centers, so the fluorescence yield reaches a value equivalent to that attained in the suppression of photochemical quenching and permits non-photochemical quenching to be recorded and photochemical quenching to be calculated (Quick & Horton 1984;

Schreiber 1986; Schreiber, Schliwa & Bilger 1986). This saturating flash of light allows the specific calculation of the maximal fluorescence of the dark-adapted state (F_m^0).

Following this, dark-adapted plants are moved into illuminated conditions typically in the range of 150 to 200 μmol photons m^{-2} s^{-1} for a minimum period of 15 min. When a dark-adapted leaf is transferred into the light, PSII reaction centres are gradually closed down causing the yield of chlorophyll fluorescence to fall down again progressively after an initial sharp increase. This is known as fluorescence quenching and consists of two components: i) photochemical quenching due to an increase in the rate at which electrons are taken away from PSII due to light activation and ii) non-photochemical quenching due to an increase in the efficiency of energy conversion to heat. After the calculation of the steady-state level of value of fluorescence *(F_t)*, a further saturating pulse of light is provided obtaining the value for the maximal steady-state fluorescence *(F_m'*; Figure 3. 1).

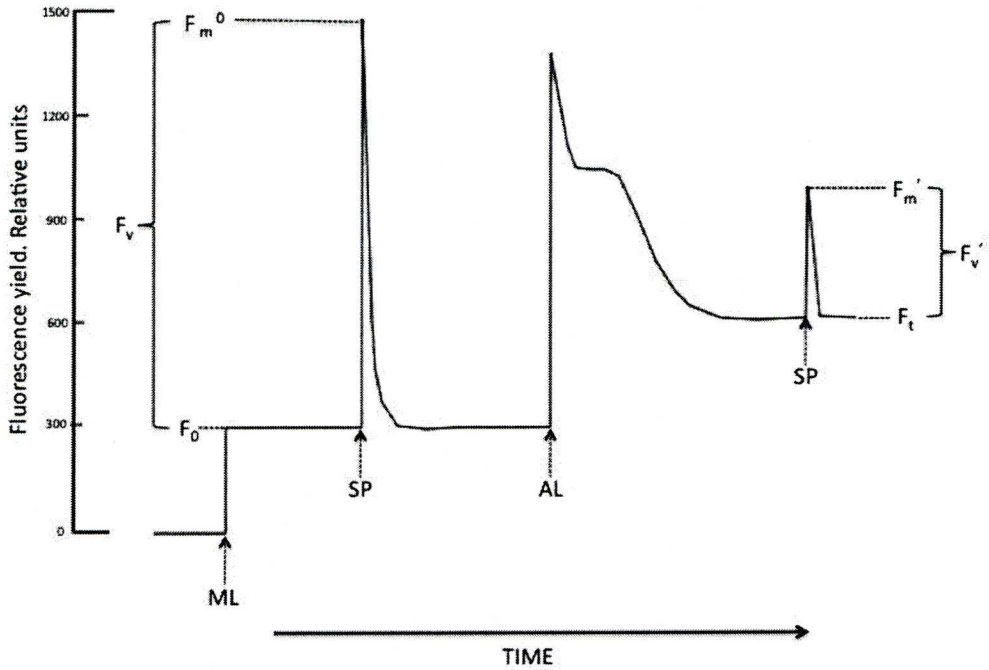

Figure 3. 1. Sequence of a fluorescence trace obtained by the saturation pulse method (adapted from Kooten and Snel 1990). A leaf is dark adapted for at least 20min. A measuring light is switched on (ML) providing the leaf with a weak measuring beam and the ground state fluorescence in the darkness is measured (F_o). Following this, a saturating pulse of light (SP) is applied and the maximum fluorescence determined in darkness by a weak measuring beam, (F_m^0). After an application of constant illumination by actinic light (AL), a temporary rise in fluorescence yield is observed. After a period of time of at least 15 min, steady state fluorescence is then measured (F_t) after which the leaf is supplied with another saturating pulse of light (SP) in order to measure the maximum fluorescence in the light (F'_m).

Chlorophyll fluorescence has been the subject of a number of reviews in the past (Krause & Weis 1984; Horton & Bowyer 1990; Kooten & Snel 1990; Krause & Weis 1991; Demmig-Adams & Adams III 1992; Samson, Prášil & Yaakoubd 1999; Maxwell & Johnson 2000b), in which the correct nomenclature for the different chlorophyll fluorescence parameters has been extensively discussed. Cognisant of the fact that there is still inconsistency in the literature, this work adopts the nomenclature proposed (Kooten & Snel 1990; Rosenqvist & van Kooten 2003) as indicated in Table 3. 1.

Table 3. 1. ***Definition of chlorophyll fluorescence parameters.***

F_0	Minimal fluorescence in the dark	All PSII reaction centres are open in the dark-adapted state
F_m^0	Maximal fluorescence in the dark	All PSII reaction centres closed in the dark-adapted state. All non-photochemical quenching processes are at a minimum
F_t	Steady state fluorescence	Fluorescence intensity in the light-adapted state with all PS II reaction centres open
F_m'	Maximal fluorescence in the light	Fluorescence intensity with all PSII reaction centres closed in the light-adapted state
F_v	Variable fluorescence in the dark	Variable fluorescence in the state when all non-photochemical processes are at a minimum ($F_m^0 - F_0$)
F_v'	Variable fluorescence in the light	Variable fluorescence in the light adapted state
ϕ_{PSII}	Quantum yield of PSII	$(F_m' - F_t) / F_m'$
F_v / F_m^0	Maximum quantum yield of PSII	$(F_m^0 - F_0) / F_m^0$
NPQ	Non-photochemical quenching	$(F_m^0 - F_m') / F_m'$

Comparison of steady-state chlorophyll fluorescence data with instantaneous light response curves can be a useful tool in ecophysiology. Rascher, Liebig et al. (2000) have argued that measurement of photosynthetic light-response curves permits a detailed characterization of photosynthetic responses, which are not related to the instantaneous incident light conditions, but rather to the ontogeny of a leaf. Moreover, examination of non-steady-state light-response curves, and the careful interpretation of the parameters obtained, can be an enormous advantage to ecophysiological studies because they permit detailed understanding of how rates of CO_2 fixation and transpiration vary with incident light providing important plant physiological parameters used in modelling photosynthesis.

In summary, the objectives of the work reported in this Chapter were to:

i) Assess steady state fluorescence to explore any variation in the photosynthetic capacity or disruption in the electron transport system of the mutant and wild-types as well as to estimate potential differences in instant photosynthetic capacity amongst genotypes through the quantification of chlorophyll fluorescence (Genty, Briantais & Baker 1989; Edwards & Baker 1993; Maxwell & Johnson 2000b);

Previous studies of this gene (Von Groll, Berger & Altmann 2002; Schlüter *et al.* 2003; Alwerdt *et al.* 2006) support our decision to use this mutant in this study; suggesting that any pleiotropic effects are not severe enough to alter the photobiochemistry of the mutant. We acknowledge however, that often there are multiple genes and biochemical pathways responsible for the phenotype. It is thus important to assess if photosynthetic activity is altered in the mutants, by their biochemical capacity and we did this by exploring chlorophyll fluorescence.

ii) Estimate potential differences in net photosynthetic rate in response to incident light intensity between the mutant and its wild-type to assess to what degree altered stomatal density affects carbon gain. and water loss in response to stomatal opening due to differential light input.

3. 2. Materials and methods

3. 2. 1. Steady-state fluorescence

A. thaliana genotypes *sdd1-2*, *col-5* and *col-0* were grown in a Conviron PGR14 plant growth chamber at a temperature of 21 °C during the day and 19 °C during the night with 50% relative humidity and 175 μmol m^{-2} s^{-1} photosynthetic photon flux density PPFD. Plants were grown from seed in individual flats containing a mixture (3:1) of SunGro Metro-mix 300 series and sand under well-watered conditions until phenotypic growth stage 6.00 where first flower is visible (Boyes *et al.* 2001a). Recording of the fluorescence emitted from chlorophyll a molecules located in the chloroplast of leaves is a widely used non-destructive tool in photosynthetical research and here we have measure it using a Mini-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Description of the design and application of this instrumentation has been published elsewhere (Schreiber 1986; Schreiber, Schliwa & Bilger 1986; Bolhar-Nordenkampf *et al.* 1989).

Photochemical parameters were calculated as follows:

a) The photochemical efficiency of photosystem II (ϕ_{PSII}), (also known as the Genty parameter, or the portion of the absorbed light used in photochemistry) from F'_m and F_t following (Genty, Briantais & Baker 1989) as

$$\phi_{PSII} = (F_m' - F_t)/ F_m' \qquad (1)$$

b) The quantum efficiency if the reaction centers at PSII were open (F_v/F_m) (Kitajima & Butler 1975; Genty, Briantais & Baker 1989)

$$F_v/F_m = (F_m^0 - F_0)/ F_m^0 \qquad (2)$$

c) Non-photochemical quenching (NPQ), which provides an indication of heat dissipation capacity for dark-adapted leaves (Bilger & Björkman 1990):

$$NPQ = (F_m^0 - F_m')/ F_m' \qquad (3)$$

3. 2. 2. Light response of photosynthetic parameters

Under the same growth conditions as described above for measurement of steady-state responses, a single leaf of *sdd1-2* and *col-5* was individually placed inside the gas-exchange chamber of a portable infrared gas analyzer (LI-COR 6400 IRGA with an integrated 6400-40 leaf chamber fluorometer, LI-COR, Inc., Lincoln, NE, USA). This allowed simultaneous measurements of gas exchange and chlorophyll fluorescence over a range of irradiances sequentially stepped down from high to low (2000, 1500, 1000, 750, 500, 400, 300, 250, 200, 150, 100, 50 and 0 μmol m^{-2} s^{-1} PPFD). Prior to measurement under irradiance, measurements of dark respiration rates and dark-adapted chlorophyll fluorescence were taken. A fan system within the leaf chamber avoided leaf heating at higher irradiances keeping leaf temperature constant at 25 °C.

The LI-COR 6400 also allows measurement of the maximum electron transport rate $(ETR/4)_{max}$ for each molecule of CO_2 assimilated. This value complements the CO_2 assimilation response curve to incident light, because electron transport rate is also

dependant on incident light (Flexas, Escalona & Medrano 1999), being very similar to the one described for light dependent CO_2 assimilation (Harbinson & Foyer 1991). So being in relation to the values obtained through photosynthesis. This measure will serve as a basis for comparison and validation of the data obtained in the light response curve.

Estimates of ϕ_{PSII} and F_m'/F_v were also calculated from data obtained with the Licor 6400 as described for the data obtained with the Mini-PAM, (Heinz Walz GmbH, Effeltrich, Germany) and reported the response of these parameters in relation to incident light within the same illumination range studied for the light response curve described above. This served me both as a basis for validation of these parameters as measured with the fluorometry analysis using the Mini-Pam system and to address differences in their response to incident light.

3. 2. 3. Analysis

Steady-state assimilation rate of CO_2 by a leaf increases as incident light increases. This increase is asymptotic up to a point known as the light compensation point (where there is insufficient light to compensate for respiratory carbon loss produced by photorespiration and dark respiration). This response may be described by a modified rectangular hyperbola rectangular model (Ye 2007). This model plots net photosynthetic rate against Photosynthetically-active radiation (PAR) fitted to:

$$P(I) = \alpha ((1-\beta I) / (1+\gamma I)) (I-R_d) \quad (4)$$

where P (I) is the net photosynthetic rate, I is PAR, R_d is the dark respiration rate, α is the initial slope of the photosynthetic light-response curve when I equals zero, and β and γ are coefficients.

Graphically, the intercept of the function with the x-axis of incident radiation ($\mu mol\ m^{-2}\ s^{-1}$) is the light-compensation point (I_c). The initial slope of the function (α) is also referred to as the apparent quantum yield (ϕ) (Lambers *et al.* 2008). The intercept with the y-axis, graphically represents the rate of dark respiration (Rd). At low irradiance, the rate of CO_2 assimilation is light-limited; at higher irradiance as the curve reaches the light saturation point (I_m), photosynthesis is carboxylation limited. P_{max} is the

light-saturated rate of CO_2 assimilation at ambient CO_2. Model fitting was completed using Table Curve 2D v5.01 (Brown 2002).

Unlike measures of photosynthetic rates that adjust rapidly to variations in ambient CO_2 pressures (C_a), measurement of stomatal conductance (g_s) takes longer to respond to variation in partial preassures of ambient CO_2, and its measurement is not as accurate (Brodribb *et al.* 2009). Keeping this source of error in mind, we studied the variation of transpiration and water use efficiency (WUE) with the incident PAR. WUE was calculated as the ratio between carbon fixed and water lost (Sinclair, Tanner & Bennett 1984; Lambers *et al.* 2008).

Differences among treatments were analysed by one-way ANOVA using the statistic program package R (R Development Core Team 2010).

3. 3. Results

Measurements of minimal level of fluorescence (F_0) and maximal fluorescence in the dark-adapted state (F_m^0), steady-state level of value of fluorescence (F_t) and maximal steady-state fluorescence (F_m') are given in Table 3. 2. There were no significant differences among genotypes in these parameters (Table 3. 2).

Table 3. 2. Estimates of chlorophyll fluorescence parameters for genotypes of *col-0*, *col-5* and *sdd1-2* as obtained from measurements conducted with the mini-PAM system. Results are given as the mean ± SE. Different letters mean significant differences.

	col-0	***col-5***	***sdd1-2***
F_0	242.57 (± *4.74*) *a*	271 (± *12.69*) *a*	244 (± *8.41*) *a*
F_m^0	1371.57 (± *58.71*) *a*	1485.17 (± *23.84*) *a*	1430.5 (± *52.8*) *a*
F_t	322.25 (± *14.65*) *a*	326.33 (± 8.08) *a*	318.33 (± *5.15*) *a*
F_m'	1200.37 (± *26.04*) *a*	1213.83 (± *11.63*) *a*	1186.5 (± *12.79*) *a*
F_v	1130	1214.17	1186.5
F_v'	878.12	887.5	868.17
ϕ_{PSII}	0.7315	0.7312	0.7317
F_v/F_m^0	0.82	0.82	0.83
NPQ	0.14	0.22	0.21

Table 3. 3. One-way ANOVA table. Statistically significant differences (one-way ANOVA, $p < 0.05$)

Chlorophyll fluorescence parameter	Source	df	SS	MS	F value	P
F_0	**Line**	2	2994.2	1497.1	3.1648	0.073
	Residuals	14	6622.7	473		
F_m^0	**Line**	2	41819	20909	1.4971	0.257
	Residuals	14	195526	139.66		
F_t	**Line**	2	192	96	0.1105	0.869
	Residuals	17	14768.2	868.7		
F_m'	**Line**	2	2242	1121	0.4061	0.6726
	Residuals	17	46922	2760		

Equally, the quantum yield of PSII (ϕ_{PSII}) the maximum quantum yield of PSII (F_v/ F_m^0) and non-photochemical quenching (NPQ) did not significantly differ between *col-0*, *col-5* and *sdd1-2*.

Figure 3. 2 illustrate the light response curves for *col-5* and *sdd1-2*, fitting equation 4 Model fits gave coefficients of determination (r^2) of 0.997 and 0.993 for *col-5* and *sdd1-2* respectively (Table 3. 4).

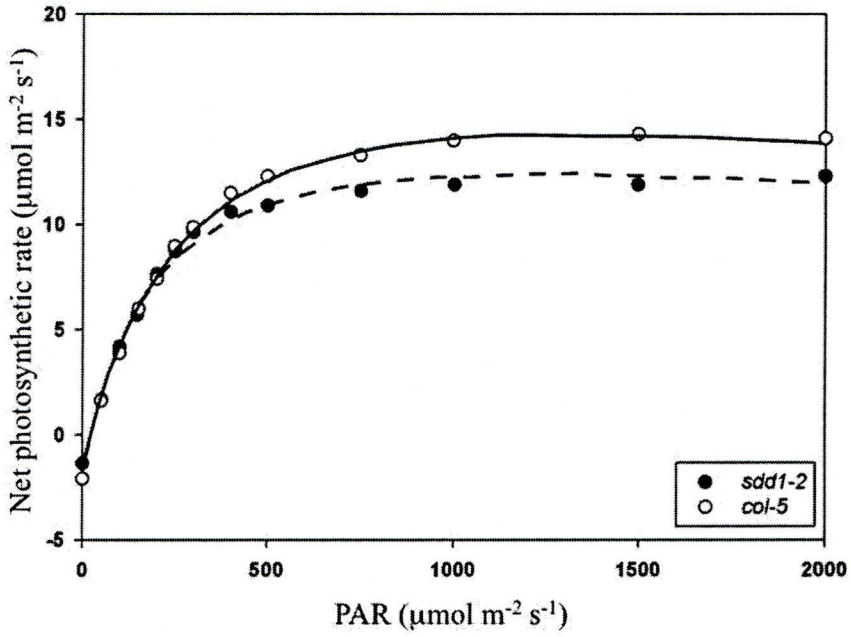

Figure 3. 2. Photosynthetic light-response curves of *col-5* and *sdd1-2* modelled according to Equation (4) in the text.

Table 3. 4. Photosynthetic light-response curves parameter estimates and model fit according to Equation (4).

Parameter	***col-5***	***sdd1-2***
Light saturated photosynthetic rate (P_{max}) (μmol m^{-2} s^{-1})	14	12
Light saturation point (I_m) (μmol m^{-2} s^{-1})	1000	750
Light compensation point (I_c) (μmol m^{-2} s^{-1})	25	25
Dark respiration rate (R_d) (μmol m^{-2} s^{-1})	27.13	19.78
Initial slope (α)	0.079	0.084
β	-0.0001	-0.0001
γ	0.004	0.005
r^2	0.9986	0.9930
p	<0.01	<0.01

The wild-type showed a higher light saturated photosynthetic rate, light saturated rate and dark respiration rate (P_{max} = 14 μmol m^{-2} s^{-1}; I_m = 1000 μmol m^{-2} s^{-1}; R_d = 27.13)

compared to *sdd1-2* (P_{max} = 12 μmol m^{-2} s^{-1}; I_m = 750 μmol m^{-2} s^{-1}; R_d = 19.78). The light compensation point (I_c) was similar in both mutants (Table 3. 4).

Figure 3. 3 illustrate the stomatal conductance (g_s) response to incident light intensity. At low incident light intensities this was markedly different between the *sdd1-2* and *col-5* mutants. As light intensity increased, maximum conductance in *sdd1-2* was reached sooner than in *col-5* at *circa* 250 mmol m^{-2} s^{-1}. Above 500 PAR, conductance rates were similar in both genotypes.

Differences in net photosynthetic rate and g_s translated into higher water use efficiency in the wild-type compared to *sdd1-2* (Figure 3. 4). The wild-type exhibited a higher water use efficiency (WUE), of over 80 μmol $mol^{-1,}$ than the stomatal density mutant *sdd1-2* when PAR was greater than 400 mmol m^{-2} s^{-1}. At lower PAR, the WUE of *col-5* exceeded *sdd1-2* except at very low PAR. Both genotypes reached their respective saturation points at approximately the same range of illumination.

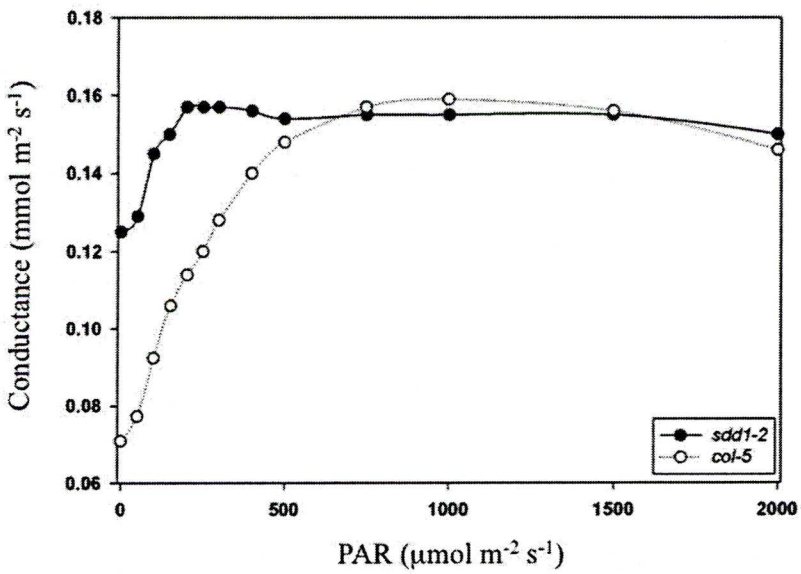

Figure 3. 3. The relationship between stomatal conductance and PAR for the genotypes *sdd1-2* and *col-5*.

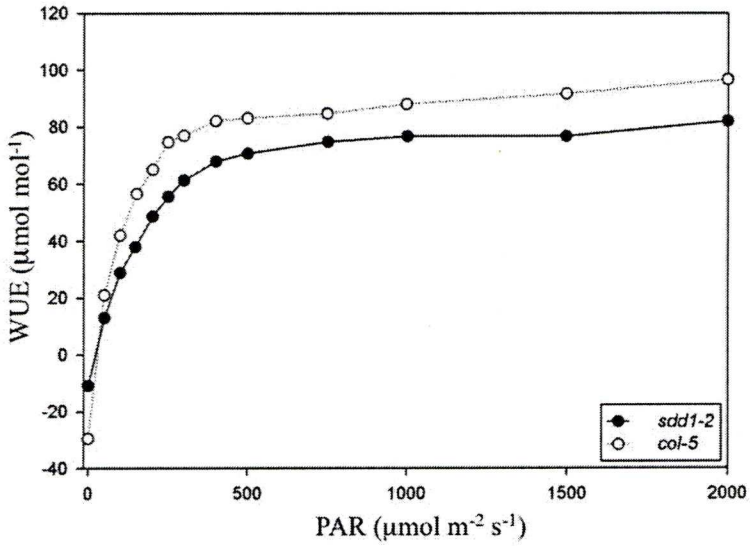

Figure 3. 4. WUE (calculated as the ratio between carbon fixed and water lost) in relation to incident light for *sdd1-2* and *col-5*.

Figure 3. 5 examines the inter-relationships between particular chlorophyll fluorescence parameters and PAR, conductance and net photosynthetic rate to comparatively assess the photosynthetic efficiency of mutant and wild-type. By examining these relations, we relate the fluorescence parameters studied at steady-state within a range of irradiance, photosynthetic rates and stomatal conductance. At elevated PAR the maximal electron transport rate $(ETR/4)_{max}$ of the wild-type exceeded that of *sdd1-2* (Figure 3. 5A). The quantum yield of PSII was consistently slightly higher in the wild-type in comparison with the high stomatal density mutant (Figure 3. 5B) across the PAR range of 0 – 2000 µmol m^{-2} s^{-1}. F'_m/F_v values noticeably declined with the increase in conductance in *sdd1-2* whereas a decline in the ratio for *col-5* was only apparent at high levels of conductance. (Figure 3. 5C).

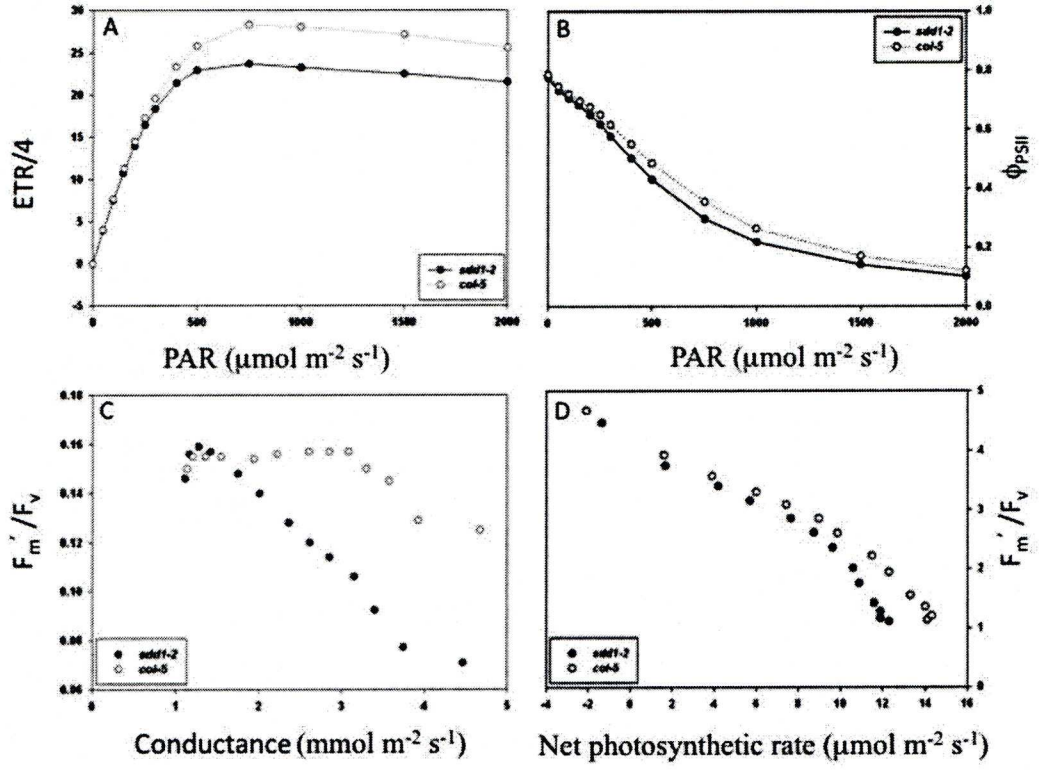

Figure 3. 5. Relationships between A). electron transport rate and incident light, B). quantum yield and incident light, C). F'_m/F_v and conductance and D). F'_m/F_v and net photosynthetic rate.

3. 4. Discussion

The principal objective of the work reported here was the comparative assessment of the photosynthetic characteristics of *sdd1-2* in comparison to its wild type. Whilst *sdd1-2* constitutes a single point mutation affecting stomatal density (Chapter 1), phenotypic variation may also be the result of pleiotropic genetic effects. Assessment of the photobiology of mutant and wild-type is fundamental in this respect given that alterations in any metabolic processes influencing photosynthetic efficiency may manifest themselves in different growth rates and changes in fitness.

In healthy leaves, the ratio F_v/ F_m^0 is always close to 0.8, (Björkman & Demmig 1987; Johnson *et al.* 1993). A value less than 0.8 indicates that a proportion of PSII reaction centers are damaged, a phenomenon called photo-inhibition, which is often observed in plants under stress conditions (Brestic *et al.* 1995). Previously, Schlüter *et al.*, (2003) showed that steady-state measurement of similar photochemical parameters of the *sdd1-1* indicated that electron transport rates did not differ between mutant and wild-type, with the further implication that *sdd1-1* and *sdd1-2* mutants may have similar photosynthetic responses. From the analysis of chlorophyll fluorescence in *sdd1-2* here, F_v/ F_m^0 ratios were close to 0.8 (Table 3. 2) suggesting that photo-inhibition was not occurring at steady-state conditions in these studies. Photo-inhibition is the gradual decrease in photosynthetic rates in plants exposed to an excessive light intensity. This has knock-on effects on plant growth. The imposition of stress factors increases the potential for photo-inhibitory effects, so its appearance in steady state conditions implies the presence of stress.

It can thus be concluded that the absence of photo-inhibition under the growth conditions used, in terms of water, temperature and light regime, indicates that the growth conditions were optimal and thus that the conditions used in subsequent Chapters as the optimal/well-watered conditions were indeed optimal. Table 3. 2 indicates that no statistically significant differences were detected in the measurable chlorophyll fluorescence parameters that characterize the photobiology of the plants. Indeed the *sdd1-2* was not significantly different from either *sdd1-2* or *col-5*, although *col-5* had the highest values of all parameters. Previously, Schlüter *et al.* (2003) showed that steady-state measurement of similar photochemical parameters for the *sdd1-1* mutant indicated

that electron transport rates did not differ between mutant and wild type. Thus, a further conclusion that can be drawn from this work is that the *sdd1-1* and *sdd1-2* mutants have similar photochemical responses.

CO_2 assimilation under steady-state conditions increased asymptotically as incident light increased (Figure 3. 2). Below the light compensation point (Ic), there was insufficient incident radiation to ensure sufficient photosynthetic CO_2 fixation for compensation of the amount of carbon lost due to respiration (both photorespiration and dark respiration). In this experiment, estimates of I_c did not differ between mutant and wild type (Table 3. 3). Above the light compensation point, net photosynthetic rate increased linearly with irradiance in both genotypes. In this phase of the response curve, light is the limiting factor for electron transport and therefore CO_2 fixation. The initial slope (α) of the light response curve therefore describes the efficiency of the conversion of light to fixed carbon, a term related to quantum yield. Both slopes did not differ noticeably (Table 3. 3) suggesting similar responses in mutant and wild type, a conclusion supported by the analysis of fluorescence showing similar quantum yields. This study was carried without repetitions, so no statistically significant differences were explored.

At high incident light levels, photosynthesis becomes light saturated due to a limitation in the rate of carboxylation. From comparison of light response curves (Figure 3. 2; Table 3. 3), the calculated values of P_{max}, R_d and I_m indicate that *col-5* had a greater photosynthetic capacity than *sdd1-2*. Thus, at high irradiance, the increased stomatal density associated with the *sdd1-2* mutant resulted in a reduced capacity for photosynthetic CO_2 fixation relative to the *col-5* wild type. This could be interpreted as suggesting that the shape of the conductance curves indicate differences in the stomatal behaviour of the wild type and mutant under high light conditions. However, at a lower irradiance of 175 $\mu mol\ m^{-2}\ s^{-1}$ including those at the range characteristic of steady-state conditions, photosynthetic performance was very similar in both wild type and mutant.

The calculated values of $(ETR/4)_{max}$ (Figure 3. 5. A), support the observation from the light response curve that *col-5* achieves a higher photosynthetic electron transport capacity. Maximum rates of electron transport were achieved earlier, at a lower

level of PAR, in *sdd1-2* than in the wild type, suggesting a difference in the photosynthetic processes of the genotypes.

Whilst differences between wild type and *sdd1-2* were found in relation to response to incident radiation, no differences were found in photobiological traits. Possible explanations for this may be that i) chlorophyll fluorescence was made at a light intensity in which net photosynthetic rate did not differ between wild-type and mutant; ii) light response curves obtained in this study were designed to identify the light intensity at which light saturation was reached, which was important in order to explore the A/Ci curve data that are described in the following Chapter; in this curves describing the response of net photosynthetic rates against the partial pressure of CO_2 at the intra-cellular level, are commonly studied at light saturation conditions in order to assume no stomatal limitation of CO_2 uptake (Farquhar & Von Caemmerer 1982; Sharkey *et al.* 2007). iii) even though a good relation between chlorophyll fluorescence values and CO_2 assimilation rates has been reported for C4 plants such as maize (Edwards & Baker 1993), this relationship has not been established in C3 plants, because photorespiration competes with CO_2 fixation for the energy harvested through photosynthetic electron transport (Cornic & Briantais 1991; Flexas *et al.* 2002).

The wild type exhibited a higher water use efficiency (WUE), of over 80 μmol $mol^{-1,}$ than the stomatal density mutant *sdd1-2* when PAR was greater than 400 μmol m^{-2} s^{-1}. At lower PAR, the WUE of *col-5* exceeded *sdd1-2* except at very low PAR. Both genotypes reached their respective saturation points at approximately the same light intensity (Figure 3. 4). The relation between genotypes with respect to carbon fixation was inverted in terms of WUE, due to the much higher stomatal conductance (g_s) found in the stomatal density mutant. We can conclude that since the diffusion coefficient of water vapour in air is 1.6 times greater than for CO_2; under well-watered conditions, plants with increased stomatal numbers present a higher capacity to uptake more CO_2 specially as stomata open along with an increased photosynthetic active radiation.

An important goal of this study was to understand the effect of differential stomatal density on plant growth. On one hand, it may be predicted that plants with higher stomatal numbers will increase relative growth rates simply because they are fixing more carbon (Figure 3. 2). By contrast, those plants also have higher

stomatal conductance (Figure 3. 3), leading to a lower water use efficiency (Figure 4. 4). Plants can adjust their phenology depending on the environmental conditions, particularly water availability. It was therefore interesting to study the effect of this trade-off between carbon gain and water loss at differential stomatal density on plant growth under optimal conditions compared to water-limited conditions. These relations are described in Chapter 5.

Chapter 4. Photosynthesis, water use efficiency and growth in genotypes *sdd1-2* and *col-5*.

4. 1. Introduction

As discussed in the introductory Chapter, stomatal pores link intercellular leaf air spaces to the atmosphere, a morphological characteristic that has major implications for physiological processes and plant fitness. This continuity allows carbon dioxide to reach the plant's mesophyll chloroplast for photosynthetic carbon fixation (Taiz & Zeiger 2006). However, plants cannot access the necessary CO_2 for photosynthetic activity without simultaneously losing water due to transpiration. This is even more problematic due to the fact that the diffusion rate of water vapour is 1.6 times greater than that of CO_2 (Hetherington & Woodward 2003). Stomatal aperture then becomes a compromise between conservation of water and maximization of CO_2 fixation (Willmer & Fricker 1996).

The number, distribution, size, shape and ability to open and close stomata on leaves are species specific, although they vary with environmental conditions (Larcher 1995; Bergmann 2004). Stomatal density plays an important role in the water use efficiency of higher plants (Woodward 1987; Woodward & Bazzaz 1988; Mansfield, Hetherington & Atkinson 1990). Stomatal closure can serve as a rapid and effective drought-avoidance response. However, prolonged stomatal closure is not sustainable, as stomatal CO_2 uptake is prevented and will limit photosynthetic assimilation and growth (Farquhar & Sharkey 1982; Schulze 1986; Valladares & Pearcy 1997; Juenger *et al.* 2005).

In the absence of variations in stomatal size among plants, stomatal density will determine the maximum stomatal conductance (g_s) that a leaf presents per unit area (Drake, Gonzalez-Meler & Long 1997a). Stomatal conductance is optimized over the long-term through developmental changes in stomatal density. Density of stomata affects the response of plants to drought conditions (El-Sharkawy, Cock & Hernandez 1985). According to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC 2007), the amount of carbon dioxide in the atmosphere in 2005

(379 ppm) is considerably larger than the natural range of the last 650,000 years (180 to 300 ppm). It seems logical that a decrease in stomatal density will lead to a decrease in stomatal conductance and will allow plants in environments where water is a limiting resource to decrease their transpiration rates and still maintain conductance for gas exchange at the leaf surface due to this increase of atmospheric CO_2 concentration and thus, avoid photosynthetic limitation. However, it is not as yet evident what the role and impact of changes in stomatal density will be, in environments with elevated CO_2 where water is not a limiting factor.

In all plant species, the stoma is an epidermal structure that is formed by two guard cells surrounding a pore whose width is physiologically regulated. Guard cells in *A. thaliana*, as in other dicots, are kidney-shaped cells whose shape is altered by changes in cell turgor, thus affecting pore width. In wild-type genotypes, guard cells are paired and face each other (Nadeau & Sack 2002). The key factor determining stomatal diffusion resistance to H_2O and CO_2 exchange is the width of the stomatal pore. Resistance to gas diffusion increases exponentially with a reduction in pore width. Stomatal conductance is directly proportional to pore width (Larcher 1995),

This Chapter presents data from a series of experiments designed to gain an understanding of the influence of altered stomatal density on photosynthesis and plant growth and physiology under differential watering, by comparing the *sdd1-2* mutant with its wild-type, *col-5*. The first objective was to measure photosynthetic activity in mutant and wild-type. As a consequence of possible changes in biochemical capacity, diffusion of CO_2 from air to the mesophyll, or stomatal function due to the presence of paired stomatal units and clusters, it can be postulated that photosynthetic capacity may be altered in *sdd1-2* with consequences for growth and fitness. In addition, this Chapter presents data comparing the growth rates and water use efficiency of the two genotypes during the vegetative stage of growth.

4. 1. 1. Photosynthetic analysis

The literature reveals that measured photosynthetic rates obtained for *A. thaliana* vary considerably between labs using the same genotype. These differences have been argued to be due to different growth conditions and genetic background (Lake 2004), and also

due to the fact that whole plant gas exchange chambers have commonly been used (Donahue, Poulson & Edwards 1997; Sun, Okita & Edwards 1999; Dodd, Parkinson & Webb 2004; Tocquin & Perilleux 2004) .

Measures of photosynthetic parameters in *A. thaliana* using whole plant measurements have been argued to present a series of problems such as leaf shading due to overlapping leaves, simultaneous measurements of leaves with different ages, and acclimation histories. There is also experimental error associated with CO_2 emissions from the soil, which has only been recently corrected with the introduction of the LICOR 6400-17 Whole Plant Arabidopsis Chamber. For example studies using the Columbia ecotype obtained results ranging from 3.5 to 9 μmol $m^{-2}s^{-1}$ when determined with whole plant chambers (Dodd, Parkinson & Webb 2004; Poulson, Boeger & Donahue 2006).

Such issues are not a concern when making single leaf measurements. Most gas exchange analyses with single leaves use commercially available infrared gas analysis systems equipped with cuvettes typically designed to enclose between 2 and 10 cm^2 of leaf surface (Long & Bernacchi 2003). However, problems derived from the small leaf area and short petioles in *A. thaliana* make leaf gas exchange difficult to measure in single leaves of *A. thaliana* (Schlüter *et al.* 2002; Schlüter *et al.* 2003). Initially, measurements in *A. thaliana* single leaves were conducted using specially designed open gas exchange system for small leaves (Muschak *et al.* 1997; Muschak, Willmitzer & Fisahn 1999). More recently, the 6400-15 Arabidopsis chamber (Li-Cor, Inc., Lincoln, NE, USA) was designed for measurements of small leaves, particularly leaves which are difficult to clamp with conventional chambers due to small leaf areas, such as *A. thaliana* (Martre *et al.* 2002; Xie *et al.* 2006). This chamber has clear apertures for natural illumination of the top and bottom of the leaf. The apertures are 1.0 cm in diameter and are positioned 8.5 cm away from the main body of the IRGA. Standard leaf chambers enclose a small area of leaf surface surrounded by gaskets to seal the chamber onto the lead, so a small area of the leaf is shaded by gaskets due to the increased perimeter against area ratio compared to larger leaf chambers. This shaded area surrounds the illuminated and photosynthetically active leaf area under measurement, which is respiring. The associated experimental error becomes more important the

smaller the measured area is; this is a source of error called the "edge effect" and increases as the diameter of the chamber decreases (Pons & Welschen 2002). Another limitation associated with this type of chamber is that the light sensor is situated at a considerable distance from the leaf chamber. Finally and more importantly, this type of chamber does not have a fluorometer, so quantification of the relationship between net photosynthetic rate (A) and carbon dioxide concentration (C_i) curves is not possible under light saturating conditions.

4. 1. 2. Growth analysis

The concept of relative growth rate (RGR) was first introduced by Blackman (1919). RGR is a term linked to the characteristics of the habitat for which the species originated (Poorter & Garnier 2007) characterizing plant performance and fitness (McGraw & Garbutt 1990). Grime and Hunt (1975) studied RGR characteristics of 132 plant species in the north east region of the UK under optimal growth conditions relating them to their characteristic habitat. They found that species with high RGR were more abundant in productive habitats and species with low RGR are typical from stable unproductive habitats. High RGR's are also associated with a short life history in which much of the plant's photosynthate is directed into seeds. The potential for rapid growth allows for opportunistic exploitation of productive habitats (Grime & Hunt 1975).

Fast growing species such as *A. thaliana*, produce relatively more leaf area and more aboveground to belowground ratio, which contribute to an increased rate of photosynthesis per unit plant dry weight. Increased RGR implies a higher respiration rate per unit of dry biomass. However, expressed as a fraction of the total amount of carbon fixed per day, they use less in respiration (Lambers & Poorter 1987).

There are two approaches to the measure of relative growth rates in plants: i) the classical approach, and ii) the functional approach. According to the classical approach (Blackman 1919), RGR is calculated by dividing the plant weight at two harvests made at two points in time divided by the time difference between those two harvest. It was originally thought that RGR would be a physiological constant characteristic of species under given conditions, but RGR changes throughout the plant's life cycle (Hunt &

Lloyd 1987). Moreover although this classical approach is easy to perform, it does not account for changes in growth rate at different points in time (Causton & Venus 1981). In the functional approach (Vernon & Allison 1963; Hunt 1982), there is a solution for this limitation of the classical approach, as repeated measures through the different stages of the development of the plant are used to fit models that describe the RGR.

Both approaches has been taken in the study of *A. thaliana*: classic (Tholen, Voesenek & Poorter 2004; Abdolzadeh *et al.* 2010) and functional (Van Der Kooij & De Kok 1996; Li, Suzuki & Hara 1998; Meyer *et al.* 2004; Paul-Victor *et al.* 2010).

4. 2. Materials and methods

4. 2. 1. Photosynthesis

Genotypes *sdd1-2* and *col-5* of *A. thaliana* were grown in a growth chamber (Conviron PGR14 plant growth chamber) being planted individually in 230x176x55 mm flats containing a mixture (3:1) of SunGro Metro-mix 300 series and sand. Plants were maintained under well-watered conditions and raised to the phenotypic growth stage 6.00 where the first flower was visible (Boyes *et al.* 2001a). The chamber was maintained at 21°C during the day and 19°C during the night with 175 μmol m^{-2} s^{-1} PPFD.

For photosynthetic measurements, a leaf was placed inside the gas-exchange chamber of a portable infrared gas analyzer (LI-COR 6400 IRGA with an integrated 6400-40 leaf chamber fluorometer, Li-COR, Inc., Lincoln, NE, USA) with a red/blue LED with blue light accounting for the 10% of the total photon flux. The relative humidity in the air stream was maintained at 60% and the leaf chamber temperature at 23 °C. The airflow in the chamber was adjusted to 150 μmol s^{-1}.

Leaves were allowed to acclimate to chamber conditions to an air CO_2 partial pressure (C_a) of 450 ppm (45.6 Pa) in the leaf chamber. After this, gas exchange rates were then determined sequentially reducing C_a to 390, 250, 150 and 50 ppm (39.5, 25.3, 15.2 and 5.7 Pa). C_a was then returned to 450 ppm (45.6 Pa), and then increased sequentially at 750, 1000, 1500, and 2000 ppm (76, 101.3, 152 and 202.7 Pa). These measurements were conducted both at steady state light conditions at 200 PAR and at

saturating light conditions (1000 PAR) as observed from the light response curve described earlier. Four replicates measurements (four individual leaves from four different plants) were used per CO_2 treatment and data for each individual curve was acquired within 40 min.

Estimates of photosynthetic parameters were calculated using the model of (Farquhar, Caemmerer & Berry 1980) as modified by Farquhar & Von Caemmerer (1982) following the assumptions and model-fitting approach of Sharkey *et al.* (2007). Following this model, the biochemical reactions of photosynthesis are in either one of three steady states (Sharkey 1985): i) Rubisco limited photosynthesis, at low partial pressure of CO_2 at the sites of carboxylation in the chloroplast (C_c)where the regeneration of RuBP is not limiting and carbon fixation can be predicted by the properties of Rubisco; ii) RuBP regeneration limited, at higher C_c where RuBP is limited and is used at a constant rate; and iii) Triose phospate use limitation (TPU), when the chloroplast reactions have a higher capacity than the capacity of the leaf to use triose phosphate. Under this last scenario, increasing O_2 concentration does not inhibit Cc. The model assumes that the net photosynthetic rate is 100% of the lowest rate allowed by the three states, or biochemical conditions.

In this Chapter data is presented for maximal leaf carboxylation capacity (V_{cmx}), maximum rate of electron transport (J_{max}), the rate of triose phosphate utilization (TPU) and mesophyll conductance (g_m) as calculated from A/Ci curves (Farquhar & Von Caemmerer 1982; Bernacchi *et al.* 2002; Sharkey *et al.* 2007). These three parameters provide information on fundamental photosynthetic processes and by comparative analysis with the *col-5* wild type enable assessment of changes in the *sdd1-2* mutant's biochemistry, CO_2 diffusion and stomatal functioning.

Each of these three states above is characterized by a distinctive CO_2 response. Thus, plotting the net rate of CO_2 assimilation against C_i and modelling the response enables determination of the biochemical capacities underlying photosynthesis, and assessment of how internal and external factors affect the components of photosynthesis in both the control and stomatal density mutant.

The diffusion of CO_2 from the atmosphere (C_a) to the sites of carboxylation in the chloroplast (C_c) of C3 plants such as *A. thaliana* through the intercellular spaces (C_i); encounters three resistances (Warren 2007): i) boundary layer resistance (BLR), ii) stomatal resistance ($1/g_s$) and iii) mesophyll resistance ($1/g_m$). Mesophyll resistances consist of resistances in both liquid and gaseous phases and from here on these are referred to as conductance (g_m) rather than resistance ($1/g_m$).

From the estimation of mesophyll conductance, the partial pressure of CO_2 at sites of carboxylation (C_c) were calculated from Fick's first law of diffusion, the relation between A, C_i and C_c being expressed as:

$$A = g_m (C_i - C_c) \qquad (1)$$

where :

A is the net photosynthetic rate,

C_i is the intercellular partial pressure of CO_2,

C_c is the partial pressure of CO_2 in the chloroplast at the site of carboxylation and

g_m is the mesophyll diffusion conductance.

Unlike measures of photosynthetic rates that adjust rapidly to variations in ambient CO_2 pressures (C_a), measurement of stomatal conductance takes longer to respond to CO_2, and its measurement is not as accurate (Brodribb *et al.* 2009). A/Ci measurements were complemented with steady-state measurements of gas exchange in order to measure more accurately the stomatal conductance and water use efficiency of plants. Water use efficiency (WUE) was calculated as the ratio between carbon fixed and water lost calculated from data obtained on net photosynthetic rate and stomatal conductance (g_s), obtained with the infrared gas analyzer, LICOR 6400 (Sinclair, Tanner & Bennett 1984; Lambers *et al.* 2008).

Rosette leaf area was measured with public domain image analysis software (Rasband 2007) from digital pictures of plants at phenotypic growth stage 6 (Boyes *et al.* 2001a) characterized by the opening of the first flower.

4. 2. 2. Growth analysis

Arabidopsis thaliana plants were grown in a growth chamber planted individually in 230

x 176 x 55 mm flats containing a mixture (3:1) of John Innes #3 and sand. The chamber was maintained at 21°C during the day and 19°C during the night with 50% relative humidity and light intensity in the range of 150 and 200 μmol m^{-2} s^{-1} PPFD.

Two water regimes were applied as treatments. Initially plants in both were watered as needed keeping flats watered to conditions as close as possible to field capacity for the first two weeks. The reason for this was to avoid variation in stomatal densities due to plasticity under differential watering particularly during the juvenile stage. Stomatal density is determined by environmental conditions existing during leaf development (Larcher 1995) but is fixed after maturation of leaves. The adult phase in *A. thaliana* can be distinguished by heteroblastic age-dependent changes in morphology of leaves, and is marked by the production of serrated leaves and an increased trichome production on both surfaces. Between these two phases, there is a brief transition phase, which is defined by the production of leaves that are partially covered with abaxial trichomes (Telfer & Poethig 1994; Telfer, Bollman & Poethig 1997; Tsukaya *et al.* 2000; Berardini *et al.* 2001). Under the growth conditions used, this transition occurred around 15 to 20 days after seedling emergence.

After this transition (20 days), high water treatments (HW) were watered to soil capacity on a weekly basis, while low water treatments (LW) were watered to soil capacity on a bi-weekly basis.

In each watering regime, six destructive harvests were conducted on days 12, 25, 33, 35, 39, 42, 46 and 50 days after emergence. Separate measurements of aboveground and belowground dry biomass were conducted by placing the plant material in an oven to dry at 60°C for 48 h and weighed. Five repetitions per treatment were taken at each harvest.

The rate of flowering was measured by recording the point in time by which individual plants reached the phenotypic growth stage 6.00, the time when the first flower was visible (Boyes *et al.* 2001a).

In a separate experiment, I measured biomass at time of flowering for *col-5* and *sdd1-2* as well as another mutation available in the *SDD1* gene, *sdd1-1* with a C24 wild-type. Each treatment (both mutants with their respective wild-type grown under well-watered and water-limited conditions) was recorded as measurements of aboveground

and below-ground dry biomass by placing the above-ground and below-ground portions of the plant in an oven to dry at 60°C for 48 h and weighed. Ten replicates per treatment were taken for each treatment.

Data analysis

Relative growth rate

Relative growth rates (RGR) of vegetative biomass (rosette and root separately) of *sdd1-2* and *col-5* were calculated by fitting the linear function to the data,

$$\log_e w = \log_e (a) + kT \qquad (2)$$

where :

w is dry biomass, T is time (days after emergence),

k is the relative growth rate, expressed as mg/mg/day and

a is the y-intercept.

This linear relation has been used several times in the past to determine the relative growth rate of *A. thaliana* (Van Der Kooij & De Kok 1996; Li, Suzuki & Hara 1998; Meyer *et al.* 2004). Logarithmic transformation achieved homoscedasity of error. Regression analysis was calculated using TableCurve 5.01 (Systat Software Co., Point Richmond, CA).

The RGR growth analysis was complemented with an analysis of the biomass achieved at the point of initial flowering, using analysis of variance. This particular stage was chosen because i) this stage represents a critical phenological stage in the life history of *A. thaliana* and the biomass of the plant at this stage has been argued to be an important fitness parameter (Boyes *et al.* 2001b); and ii) it enabled comparison of biomass performance at this stage with physiological measurements taken at the same point in time as described earlier.

Flowering rate

Rate of flowering was analysed as the cumulative percentage of flowering vs. days after emergence (DAE) from the point in time where the first flower was visible on individual plants (phenotypic growth stage 6) The best-fit equation to express the relationship

between the cumulative percentage of flowering and DAE under well-watered conditions was linear:

$$y = a + bx \quad (3)$$

where :

y is the cumulative percentage of flowering,

x is time (days after emergence),

b is the flowering rate, expressed as cumulative percentage of flowers/day and a is the y-intercept.

In order to compare linear fits I performed an analysis of covariance analysis (ANCOVA); modelling weight (the response variable) as a function of either genotype (*col-5* and *sdd1-2*) or water regime (HW or LW) and time (DAE). The model therefore has four parameters: two slopes (one slope for either *col-5* or HW and another slope for either *sdd1-2* or HW) and two intercepts (one for either *col-5* or HW and another for either *sdd1-2* or HW):

The best-fit equation to express the relationship between the cumulative percentage of flowering and time after emergence under watered-limited conditions was modelled by fitting a reverse asymmetric sigmoid function; as determined using TableCurve 5.01 (Systat Software Co., Point Richmond, CA):

(4) $$y = a + b\left[1 - \left[1 + \exp\left(\frac{(x + d\ln(2^{\frac{1}{e}} - 1) - c)}{d}\right)\right]^{-e}\right]$$

where:

y is the cumulative percentage of flowering,

x is time (days after emergence),

c is the centre of the curve or DAE when 50% of plants have reached the flowering stage and a, b, d and e are model parameters. Flowering rates were calculated from the first derivative.

Statistical analysis was conducted using the statistic program package R (http://cran.r-project.org/) by a two factor ANOVA using Type III sum- of-squares. Normality of the residuals was tested with the Shapiro–Wilk test. Homogenity of variance

was tested using the Fligner-Killeen test and comparisons of means using the post-hoc Tukey pair-wise test at the $\alpha = 0.05$ level.

Regression analysis was completed using TableCurve 5.01 (Systat Software Co., Point Richmond, CA).

4. 3. Results

4. 3. 1. Photosynthesis

Measurement of net photosynthetic rate (Figure 4. 1.A) indicated that CO_2 fixation increased as a result of increasing incident light, as would be expected from a maximal aperture of stomata, but was not significantly different between the *sdd1-2* mutant and its wild type (Table 4. 1) either at steady state (200 PAR) or light saturating conditions (1000 PAR).

Stomatal conductance (Figure 4. 1B) increased as a result of exposure to light saturating conditions (Table 4. 1). Averaging over light levels, *sdd1-2* had a higher stomatal conductance rate (g_s) than the wildtype. The interaction term between genotype and light was not significantly different (Table 4. 1).

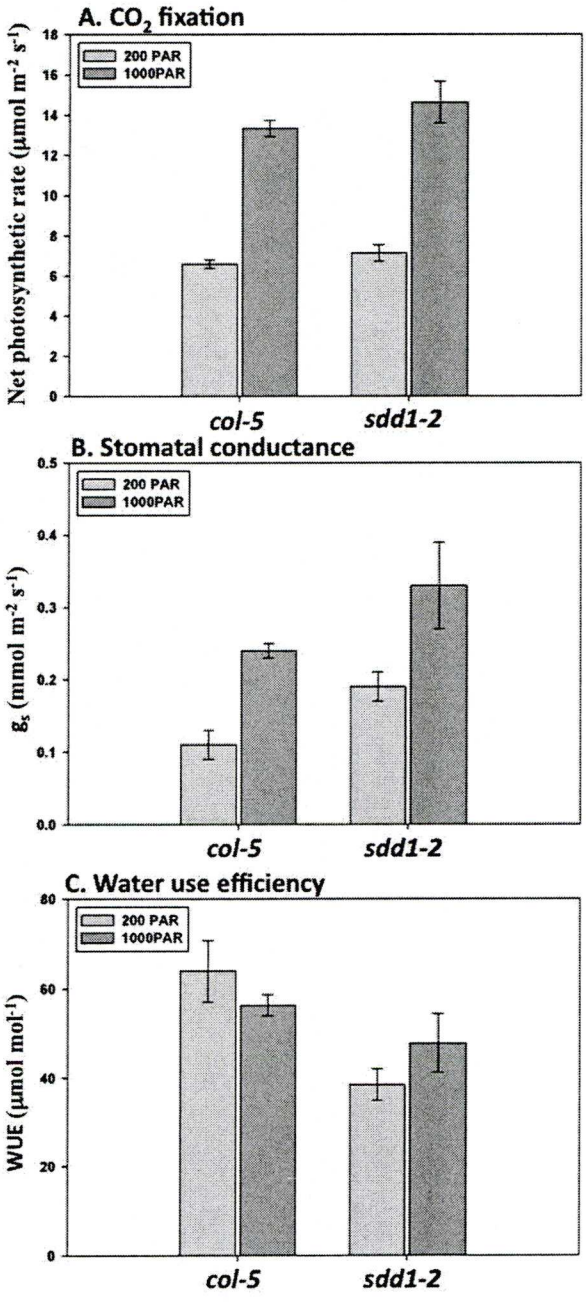

Figure 4. 1. A) Net photosynthetic rate, B) stomatal conductance (g_s) and C) water use efficiency (WUE) of *col-5* and *sdd1-2* as measured under steady-state conditions (200 PAR) and light saturating conditions (1000 PAR). Results are expressed as the mean of six replicate leaves ± SE.1

Figure 4. 1.C shows the water use efficiency of the plants (WUE), calculated as the ratio between carbon gain and water loss. Analysis of variance indicated that was no significant effect of light intensity on WUE, averaging over genotypes. Under steady-state conditions at 200 PAR, the stomatal density mutant had lower water use efficiency than the wild type *col-5* even though these differences were not significant (Table 4. 1). Conversely averaging over genotypes, *col-5* exhibited a higher WUE than *sdd1-2* (Table 4. 1). The interaction term between genotype and light was not significant (Table 4. 1)

Table 4. 1. ANOVA table for CO_2 fixation, stomatal conductance (g_s) and water use efficiency (WUE) for *col-5* and *sdd1-2* under steady-state conditions (200 PAR) and light saturating conditions (1000 PAR).

Physiological variable	Source of variation	df	SS	F value	P
CO_2 fixation rate	***Light intensity***	1	247.439	192.104	<0.01
	Genotype	1	3.825	2.969	0.104
	Light intensity x genotype	1	0.665	0.516	0.483
	Residual	16	20.609		
Stomatal conductance	***Light intensity***	1	0.084	20.545	<0.01
	Genotype	1	0.037	9.044	<0.01
	Light intensity x genotype	1	0	0.029	0.867
	Residual	16	0.066		
Watering use efficiency	***Light intensity***	1	1.67	0.012	0.912
	Genotype	1	1577.94	11.754	<0.01
	Light intensity x genotype	1	367.26	2.736	0.116
	Residual	16	2282.21	2.736	

Table 4. 2. Estimates for maximum rate of carboxylation, Vc_{max}, maximum rate of electron transport, J_{max}, the rate of triose phosphate utilization, TPU and mesophyll conductance, g_m as derived from analysis of the A/C_i curve. Results are expressed as the mean of five replicate leaves ± SE. Statistically significantly differences (ANOVA, $p < 0.005$) between groups are indicated by different letters.

	200 PAR		*1000 PAR*	
	col-5	*sdd1-2*	*col-5*	*sdd1-2*
Vc_{max}	31.75 (±2.72) *a*	29.25 (±5.01) *a*	84.2 (±8.57) *b*	71.77 (±4.21) *b*
J	47.75 (±3.12) *a*	49 (±3.87) *a*	101.4 (±4.88) *b*	100.5 (±3.71) *b*
TPU	4.45 (±0.35) *a*	4.45 (±0.35) *a*	7.06 (±0.22) *b*	7.47 (±0.4) *b*
Rd	1.65 (±0.52) *a*	1.38 (±0.54) *a*	2.42 (±0.23) *b*	2.16 (±0.14) *b*
g_m	30 (±0) *a*	30 (±0) *a*	1.02 (±0.09) *a*	1.39 (±0.25) *a*

Table 4. 2 presents the A/C_i parameters estimated from the model of Farquhar *et al.*, (1980). No statistically significant differences were observed between *sdd1-2* and *col-5*. However, increasing the incident radiation from 200 PAR to 1000 PAR translated into an increase in the maximum rate of carboxylation, Vc_{max}, maximum rate of electron transport, J_{max}, and the rate of triose phosphate utilization, TPU. Contrary to stomatal conductance, mesophyll conductance (g_m) remained unaltered as incident radiation reached saturation. As a consequence, when net photosynthetic rate was plotted against the CO_2 partial pressure at the site of carboxylation (Figure 4. 2), similar trends were seen for both genotypes.

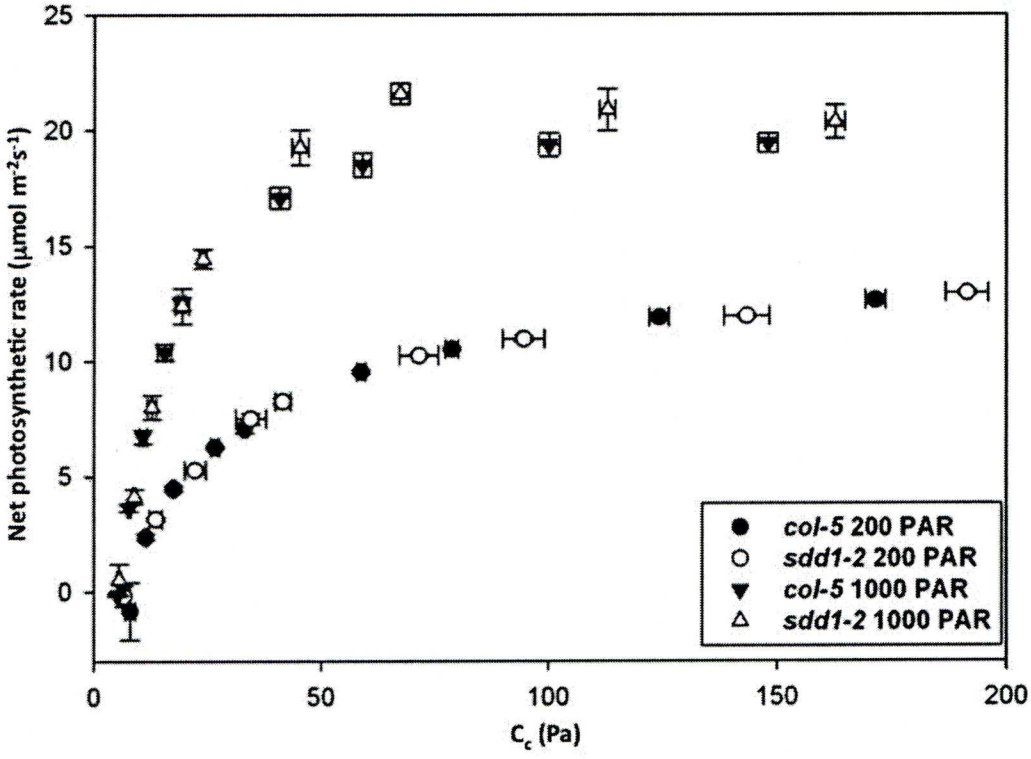

Figure 4. 2. Photosynthetic response to CO_2 partial pressure in the sites of carboxylation (C_c) at steady-state (200 PAR) and light saturating conditions (1000 PAR) in leaves of *sdd1-2* (open symbols) andwild type *col-5*, (closed symbols). Each datum is the mean ± SE (n= 5).

4. 3. 2. Growth in biomass

Relative growth rates were calculated (equation 1) from linear regression analysis taking two approaches, either by including replicate observations at each harvest or by averaging biomass estimates at each harvest. From inspection of residuals, examination of systematic lack of fit and the precision of parameter estimates it was concluded that fits based upon averaged biomass at each harvest provided more precise estimates of RGR than from regressions using the entire data set. Characteristically there was greater error variance associated with low biomass measurements even after logarithmic transformation when replicate data was included in the analysis. Results from regression analysis are therefore based on meaned observations, even though there was a loss of degrees of freedom.

Figure 4. 3 graphically illustrates the growth of the two mutants in above ground biomass under each of the two water regimes by pairwise comparison and Table 4. 3 presents RGRs after linear regression analysis. Analysis of co-variance (Table 4. 4) indicated that the growth rates of the two mutants did not differ significantly under HW (P=0.59) or under LW (P=0.06). Cross comparisons within mutants of water regimes indicated that in both mutants the growth rates were significantly different, with higher growth rates being observed under HW, the difference in growth rates (0.013 mg/mg/day) between HW and LW being the same for both mutants.

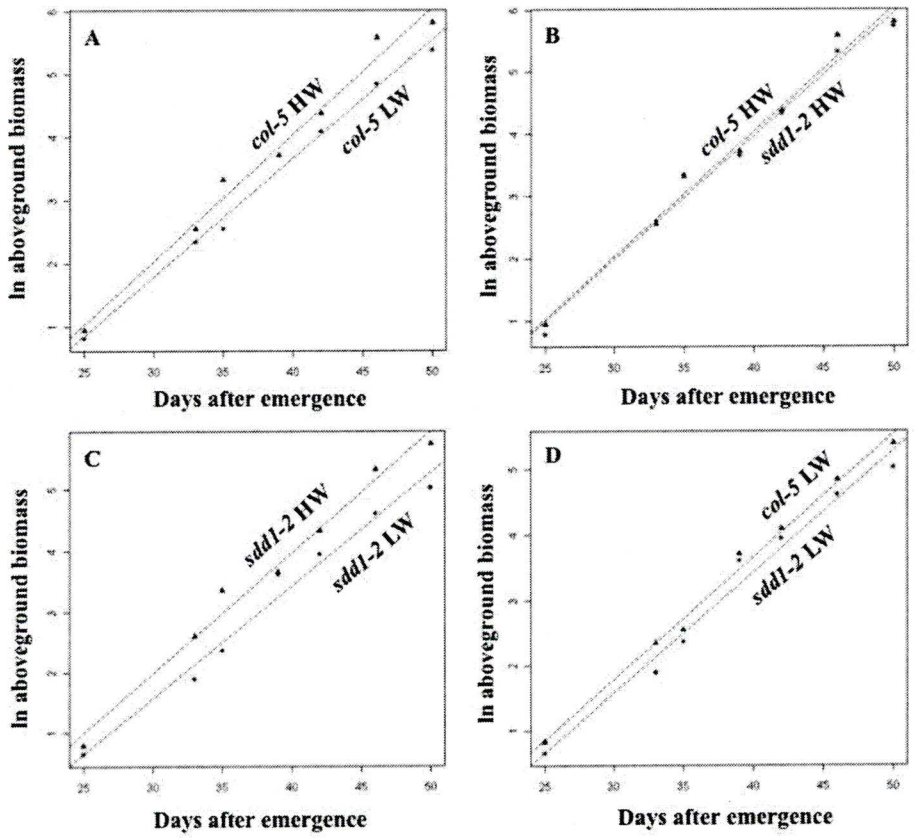

Figure 4. 3. Growth of aboveground biomass of *col-5* and *sdd1-2* in relation to time under well-watered HW and water-limited conditions (LW). Fitted line shown is from linear regression of meaned measurements at each harvest.

Table 4. 3. Estimates of relative growth rate of aboveground biomass for *col-5* and *sdd1-2*.

Treatment	Relative growth rate (mg/mg/day)	Standard error	Model fit P value	r^2
col-5 HW	0.210	0.239	<0.01	0.984
sdd1-2 HW	0.198	0.232	<0.01	0.984
col-5 LW	0.187	0.151	<0.01	0.990
sdd1-2 LW	0.185	0.256	<0.01	0.980

Table 4. 4. ANOVA table for the ANCOVA model on aboveground biomass using either genotype or water regime as a covariate for the model.

Pairwise comparison	Source of variation	df	SS	F value	P
col-5 HW vs. *col-5* LW	*Watering regime*	1	0.457	11.383	<0.01
	Time (DAE)	1	32.107	800.779	<0.01
	Water regime x DAE	1	0.039	0.98	0.346
	Residuals	10	0.401		
sdd1-2 HW vs. *sdd1-2* LW	*Watering regime*	1	0.96	16.786	<0.01
	Time (DAE)	1	31.311	547.352	<0.01
	Water regime DAE	1	0.036	0.635	0.444
	Residuals	10	0.572		
col-5 HW vs. *sdd1-2* HW	*Genotype*	1	0.017	0.31	0.59
	Time (DAE)	1	33.935	609.939	<0.01
	Genotype x DAE	1	0.002	0.027	0.872
	Residuals	10	0.056		
col-5 LW vs. *sdd1-2* LW	*Genotype*	1	0.189	4.548	0.059
	Time (DAE)	1	29.556	709.415	<0.01
	Genotype x DAE	1	0.001	0.024	0.88
	Residuals	10	0.417		

Unlike aboveground biomass, RGRs of belowground (root) was significantly stimulated by water limitation (Table 4. 5 and 4. 6) in both genotypes. Water limitation stimulated growth to a larger extent in *col-5* than in *sdd1-2* (0.053 mg/mg/day in *col-5* compared to 0.026 mg/mg/day of *sdd1-2*. The differences in growth rate between mutants under the same watering regime were very similar (~0.0135 mg/mg/day), but with the *sdd1-2* mutant outranking the wild-type under HW, the reverse being true under LW (Figure 4. 4).

Table 4. 5. Estimates of relative growth rate of belowground biomass for *col-5* and *sdd1-2*.

Treatment	Relative growth rate (mg/mg/day)	Standard error	P value	r^2
***col-5* HW**	0.111	0.152	<0.01	0.969
***sdd1-2* HW**	0.124	0.150	<0.01	0.976
***col-5* LW**	0.164	0.180	<0.01	0.980
***sdd1-2* LW**	0.150	0.153	<0.01	0.983

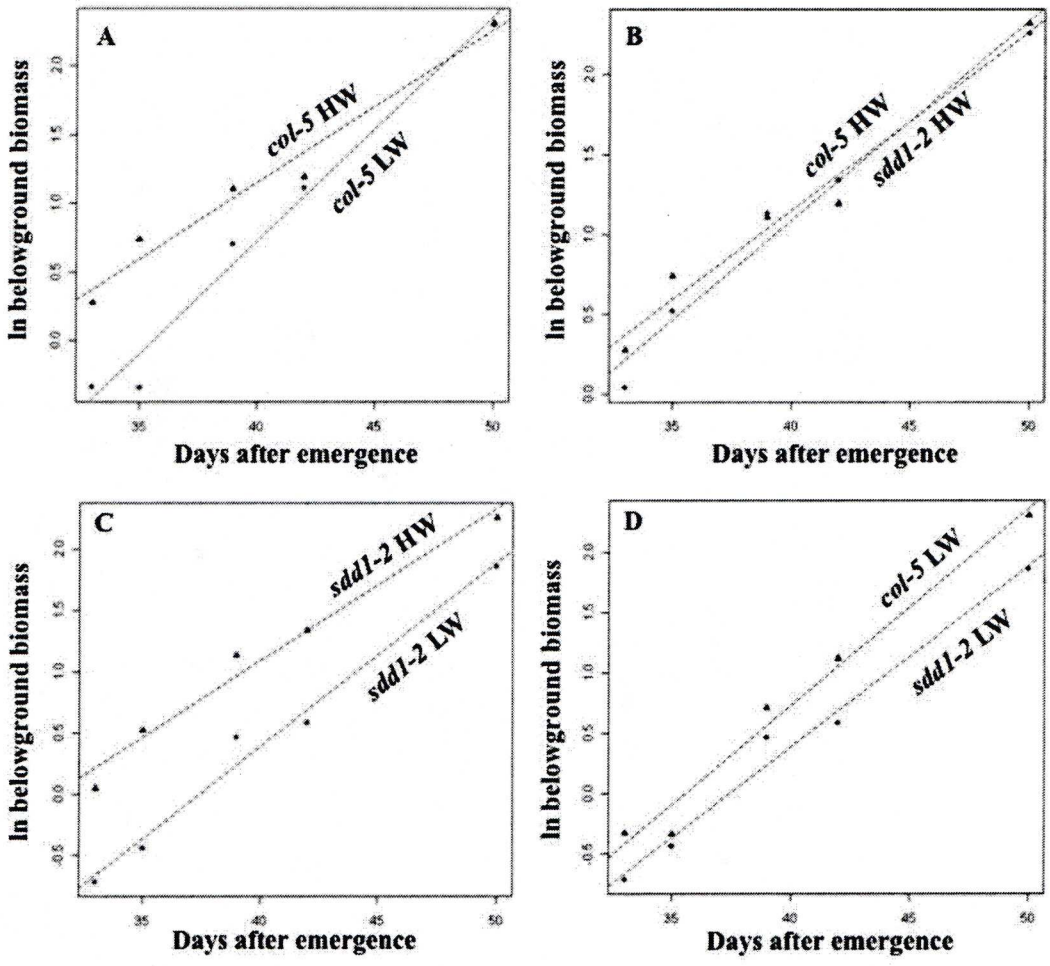

Figure 4. 4. Growth of below ground biomass of *col-5* and *sdd1-2* in relation to time under well-watered HW and water-limited conditions (LW). Fitted line shown is from linear regression of meaned measurements at each harvest.

Table 4. 6. ANOVA table for the ANCOVA model on belowground biomass.

Pairwise comparison	Source of variation	df	SS	F value	P
col-5 HW vs. *col-5* LW	*Watering regime*	1	0.474	16.983	<0.01
	Time (DAE)	1	6.7608	242.021	<0.01
	Water regime x DAE	1	0.247	8.855	<0.05
	Residuals	6	0.168		
sdd1-2 HW vs. *sdd1-2* LW	*Watering regime*	1	1.25	54.4	<0.01
	Time (DAE)	1	6.74	293.516	<0.01
	Water regime x DAE	1	0.06	2.622	0.156
	Residuals	6	0.138		
col-5 HW vs. *sdd1-2* HW	*Genotype*	1	0.011	0.496	0.508
	Time (DAE)	1	4.96	217.29	<0.01
	Genotype x DAE	1	0.015	0.677	0.442
	Residuals	6	0.023		
col-5 LW vs. *sdd1-2* LW	*Genotype*	1	0.287	10.215	<0.05
	Time (DAE)	1	8.821	314.076	<0.01
	Genotype x DAE	1	0.016	0.579	0.475
	Residuals	6	0.028		

Rate of flowering

Figure 4. 5 shows the cumulative increase in flower number as a percentage of the total number recorded by day 58 DAE of both genotypes under HW. The first visible flower of *col-5* was observed one day later (43 DAE) than in *sdd1-2* mutant (42 DAE); and both reached 100% of cumulative flowering at 58 DAE. Both genotypes attained a similar rate of flowering during that span of time and no statistically significant differences were found (rates of flowering being 5.602 % plants in flower/day for *col-5* vs. 5.421 % plants in flower/day for *sdd1-2*; Table 4. 7 and 4. 8).

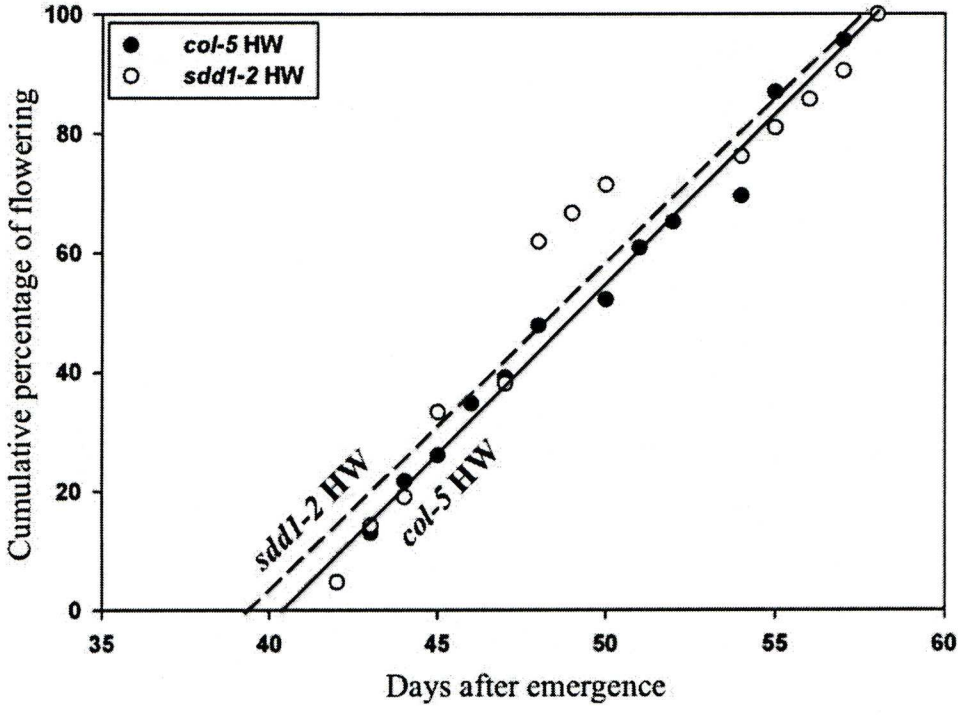

Figure 4. 5. Rate of flowering expressed as the cumulative percentage of flowering with time of *col-5* (continuous line) and *sdd1-2* (dashed line) under well-watered (HW) conditions.

Table 4. 7. Model fit (Equation 2) for cumulative percentage of flowering and time (DAE) for *col-5* and *sdd1-2* under HW using a linear fit.

Treatment	Relative flowering rate (cumulative% plants in flower/day)	Standard error	P value	r^2
***col-5* HW**	5.602	0.190	<0.01	0.987
***sdd1-2* HW**	5.421	0.458	<0.01	0.927

Table 4. 8. ANOVA table for the ANCOVA model on time of flowering for well watered treatments.

Source of variation	df	SS	F value	P
Genotype	1	34.2	0.7596	0.393
Time (DAE)	1	20449.3	454.3657	<0.01
Genotype x DAE	1	5.5	0.1215	0.731
Residuals	22	45		

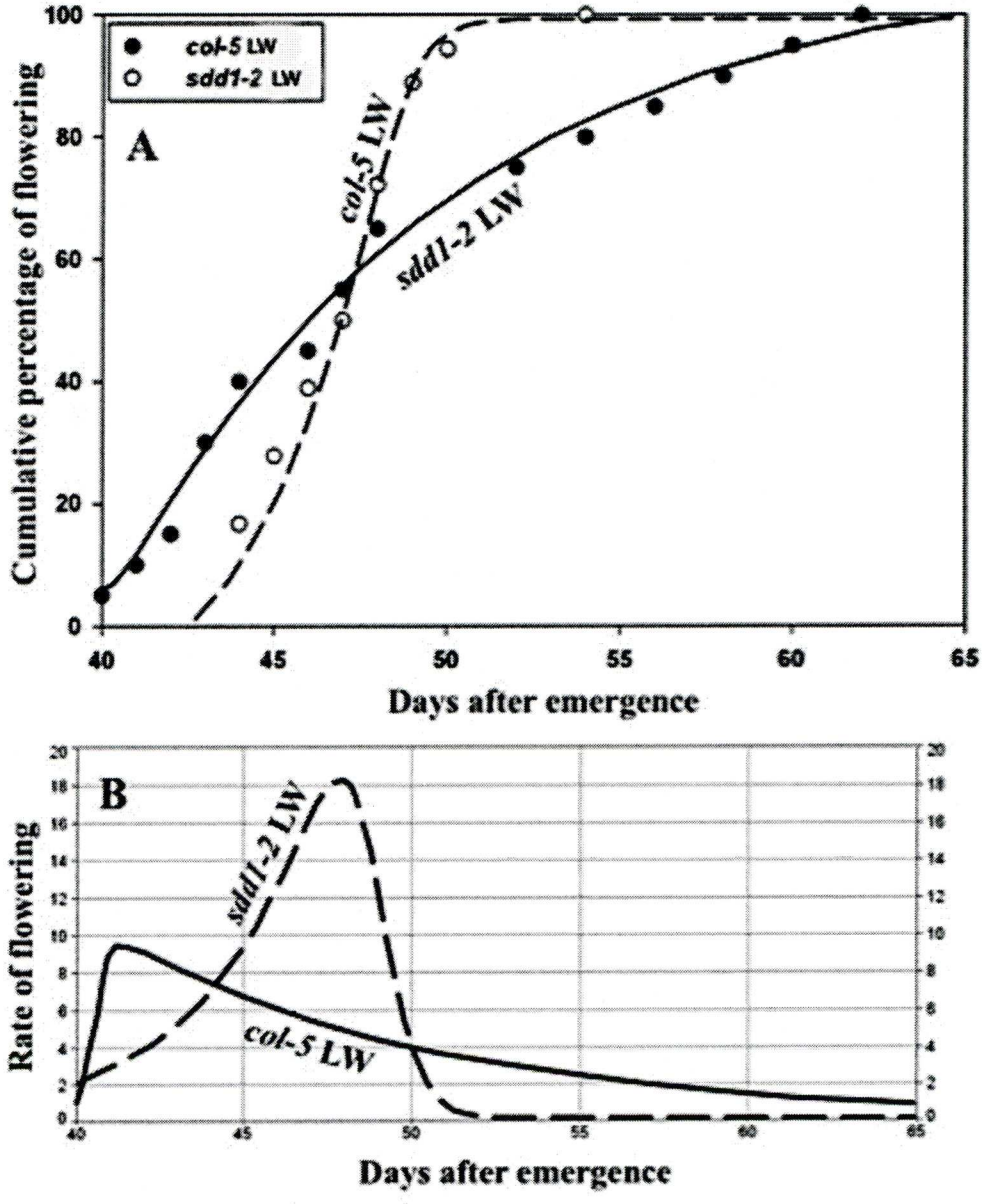

Figure 4. 6. A. Model fit for cumulative percentage of flowering and time (DAE) for *col-5* and *sdd1-2* under water-limited conditions (LW) using Equation 3. B. Change in the flowering rate with time (DAE).

Table 4. 9. Rate of flowering estimates and model fit according to Equation 3 for *col-5* and *sdd1-2* under water limited conditions based on the cumulative percentage of flowering.

Treatment	*a*	*b*	*c*	*d*	*e*	P value	r^2
***col-5* LW**	4.796	103.881	47.343	0.227	0.023	<0.01	0.992
***sdd1-2* LW**	-6.542	105.571	46.6	0.6	0.18	<0.01	0.997

The mean flowering time for *col-5* was 48 DAE while the average *sdd1-2* plant reached the flowering stage one day before (47 DAE). Figure 4. 6 and Table 4. 9 present the flowering response of the two genotypes under LW, which was non linear with time. The flowering period of *col-5* was longer, starting sooner than that for *sdd1-2* (42 DAE vs. 44 DAE in *sdd1-2*) and finished later (62 DAE for *col-5* for 54 DAE for *sdd1-2*). The peak of flowering was sooner in *col-5* (around 42 DAE) and then flowering decreased gradually whilst in *sdd1-2* flowering peaked later (48 DAE) and decreased more abruptly. Thus, under LW, in *col-5* flowering rate peaked sooner and was distributed over a time span of 10 days. Contrastingly flowering in *sdd1-2* occurred over a 20 day period, with the peak rate of flowering occurring 6 days later than in the wild-type.

Biomass at time of flowering

Biomass at time of flowering was examined in two mutant comparisons with their respective wildtypes (Figure 4. 7A). Significant differences (Table 4. 10) in above ground biomass in response to water regimes were evident at this stage of development, together with differences amongst genotypes in comparison to respective wild type. However there were no differential responses to water regimes by genotypes (genotype X water regimes terms, $P > 0.05$) in *sdd1-2* mutant compared to *col-5* wild-type. This response to water regime by genotype interaction was however significant in the other *SDD1* mutation, *sdd1-1* compared to its *C24* wild-type. It was noticeable that differences in performance at this stage both between mutant and wild type and in response to watering regime were larger in the *sdd1-2* / *col-5* comparison than for *sdd1-1* versus *C24*.

The pattern of response to watering regime in root biomass differed significantly between mutant comparisons. Whereas there were no significant differences in response to water regime by *C24* and *sdd1-1* (Table 4. 11), the LW regime reduced below ground biomass in both *col-5* and *sdd1-2* in comparison to HW. Averaging over watering regimes, *col-5* exhibited slightly larger below ground biomass than *sdd1-2*.

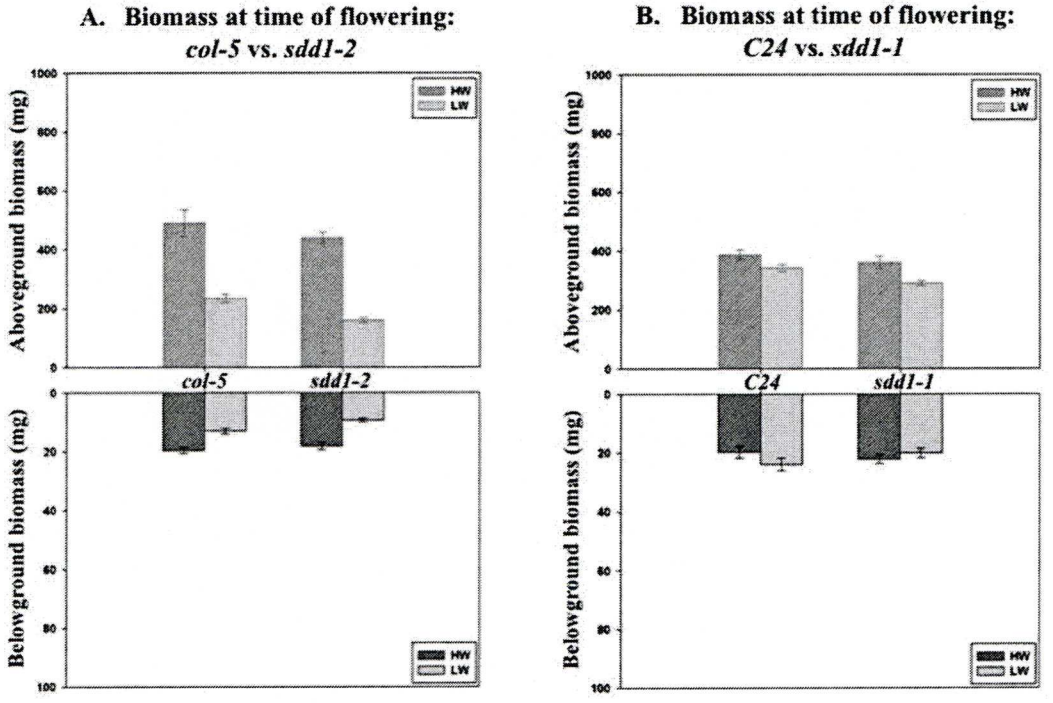

Figure 4. 7. Biomass at time of flowering for the two stomatal density mutants, *sdd1-2* and *sdd1-1* and their respective wild types *col-5* and *C24* under LW and HW watering regimes.

Table 4. 10. Two-factor ANOVA table for aboveground biomass at time of flowering for two different mutations on the SDD1 gene compared to their respective wild-types. For each comparison there were two factors: water regime and genotype. Water is a factor with two levels: high water regime (HW) and water limited or low water regime (LW). Genotype has two levels as well: stomatal density mutant (*sdd1-2* or *sdd1-1*) and their respective wild-types (*col-5* or *C24* respectively).

Pairwise comparison	Source of variation	df	SS	F value	P value
col-5* vs. *sdd1-2	***Watering regime***	1	738589	112.221	<0.01
	Genotype	1	39853	6.055	<0.05
	Water regime x genotype	1	1493	0.227	0.637
	Residual	36	236937		
C24* vs. *sdd1-1	***Watering regime***	1	16057	6.266	<0.05
	Genotype	1	17048	6.653	<0.05
	Water regime x genotype	1	13233	5.164	<0.05
	Residual	30	76872		

Table 4. 11. Two-factor ANOVA table for belowground biomass at time of flowering for two different mutations on the *SDD1* gene compared to their respective wild-types. For each comparison there were two factors: water regime and genotype. Water is a factor with two levels: high water regime (HW) and water limited or low water regime (LW). Genotype has two levels as well: stomatal dentisty mutant (*sdd1-2* or *sdd1-1*) and their respective wild-types (*col-5* or *C24* respectively).

Pairwise comparison	Source of variation	df	SS	F value	P value
col-5* vs. *sdd1-2	***Watering regime***	1	647.94	68.352	<0.01
	Genotype	1	63.90	6.741	<0.05
	Water regime x genotype	1	12.05	1.271	0.267
	Residual	37	350.74		
C24* vs. *sdd1-1	***Watering regime***	1	85.68	3.108	0.088
	Genotype	1	1.66	0.06	0.808
	Water regime x genotype	1	192.45	6.981	<0.05
	Residual	30	82703		

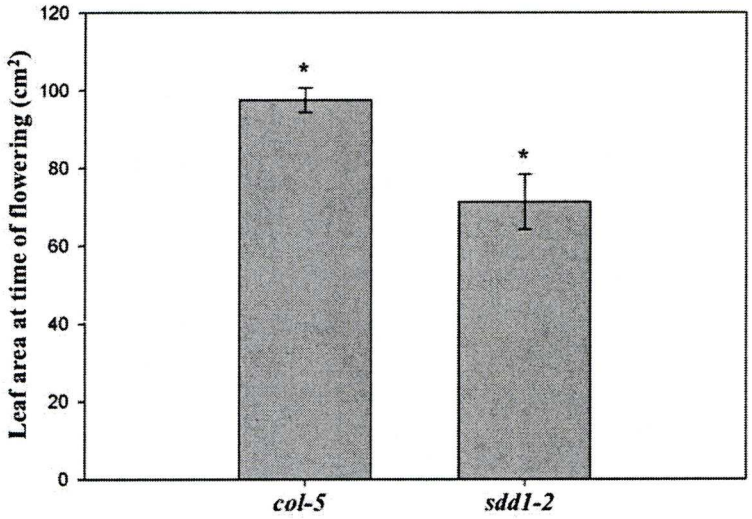

Figure 4. 8. Leaf area at time of flowering for *col-5* and *sdd1-2* under well watered conditions. Leaf area was significantly reduced ($p<0.05$) in the stomatal density mutant (*sdd1-2*) compared to its wild-type (*col-5*).

Leaf area per plant was significantly higher in the *col-5* mutant than in *sdd1-2* at the time of flowering (Figure 4. 8)

4. 4. Discussion

The causal inter-relationships amongst stomatal density, photosynthesic rate and plant growth have been extensively reviewed in crop species with the objective of improving yield by improving water use efficiency (Heichel 1971; Miskin, Rasmusson & Moss 1972; Walton 1974; Yoshida , Dale & Rasmusson 1975; Radin 1984; Araus *et al.* 1986; Heursel, Ceulemans & Ibrahim 1987; Jones 1987; Lu & Zeiger 1994; Radin *et al.* 1994; Percy *et al.* 1996; Kundu & Tigerstedt 1999; Yu 2001; Liao, Chang & Wang 2005; Yousufzai, Siddiqui & Soomro 2009). In the series of experiments reported in this Chapter the relationship between the number of stomata on the leaf and the influence that this anatomical trait has on the physiology and growth of a C3 plant, *A. thaliana* was examined by comparing the photosynthetic capacity of the *sdd1-2* mutant with its wild-type.

There are few examples in the literature of the use of single leaf

photosynthesis measurements using an IRGA leaf chamber with an integrated fluorometer allowing the determination of A/Ci curves under steady state and light saturating conditions. This is due to the fact that plants need to be grown in optimal conditions to attain leaf areas large enough to allow measurements using this type of chamber (Lefebvre *et al.* 2005; Bussis *et al.* 2006; Flexas *et al.* 2007a). The work reported here presents a further contribution to the literature on single leaf photosynthesis measurements using an IRGA leaf chamber with an integrated fluorometer. Possible changes to the photosynthetic capacity in the mutant were investigated by evaluating changes in biochemical capacity and/ or the diffusion of CO_2 through the mesophyll (Table 4. 1). Estimations of maximal leaf carboxylation capacity (V_{cmx}), maximum rate of electron trasport (J_{max}), the rate of triose phosphate utilization (TPU) and mesophyll conductance (g_m) from A/Ci curves did not show differences between the stomatal density mutant and its wild-type line among all the measured parameters. Given the similarity in biochemical capacity and diffusion of CO_2 through the mesophyll in the mutant and wild-type at an ambient growth light level and saturating light conditions; the differences previously mentioned (Figure 4. 1) in the photosynthetic activity can be argued to be solely due to stomatal regulation. This supports the findings gathered from the chlorophyll fluorescence analysis described in the previous Chapter.

This study of the photosynthetic properties of *sdd1-2* and *col-5* CO_2 fixation demonstrated that the mutant did not differ from the wild-type either at steady-state conditions (200 PAR) or at light saturating conditions (1000 PAR). At 1000 PAR, photosynthetic rate was maximized and stomatal opening peaked (Figure 4. 1.A). In plants *well*-adapted to their environment, stomata play a relatively small role in determining the rate of photosynthesis, comprising less than about 20% of the total photosynthetic limitation (Jones 1998). Previous studies under steady state conditions on the effect of elevated stomatal density in the *sdd1-1* mutant suggested that adjustments of stomatal aperture compensated for altered stomatal density (Bussis *et al.* 2006). Similarly, in the present study, photosynthetic rate did not differ between mutant and wild-type under light-saturating conditions where stomatal opening was at its maximum.

Arguably adjustments of stomatal aperture are responses to regulate water loss in order to maintain optimal water use efficiency between carbon gained and water lost.

Water loss by leaves increases as a plant is subjected to light saturating conditions as stomatal opening reaches its maximum. However whilst *sdd1-2* had an increased conductance to water, this conductance was not significantly different from that measured in the wild type. (Figure 4. 1B).

Analysis of the water use efficiency of the plants (Figure 4. 1.C) showed that maximal stomatal opening at saturating light conditions (1000 PAR) resulted in lower water use efficiencies compared to those at steady-state light conditions (200 PAR). This was probably due to the fact that under ambient conditions, regulation of stomatal opening occurred to attain an optimal compromise between carbon gain and water loss. Thus the experimental results suggest that having an elevated number of stomata results in plants with lower water use efficiency and stomatal regulation of opening does not compensate for altered stomatal density. Thus, the data presented here differ from the results of Bussis *et al.*, (2006) in *sdd1-1* and *SDD1* overexpressers. The fact that stomatal conductance was not significantly different between genotypes but WUE could be explained because of high standard errors found on the analysis of stomatal conductance and that WUE was a ratio.

As described in Chapter 3 and supported by the results of this Chapter (Table 4. 2) at saturating light intensities, CO_2 uptake which maximal rate is limited by stomatal resistance, no longer restricts carboxylation, but also the rate at which RuBP is available limits the activity of Rubisco. The rate at which this takes places, depends on the Calvin cycle, which at the same time depends on the rate at which ATP and NADPH are produced in the light reactions of photosynthesis. Photosynthetic rates are thus limited under light saturating conditions ultimately by the rate of electron transport and as we noted in the previous Chapter, since this was not different between mutant and wild type we can assume that difference in photosynthetic parameters under light saturating conditions may be due to differences in stomatal resistance between both genotypes.

In a water-limited scenario, the water economy of plant species can be compromised and thus regulation of transpiration is determined, in part, by stomatal density. Desiccation tolerance can be defined as the ability of an organism to find

equilibrium between its internal water potential and that of its surrounding environment (Alpert 2000). In the mutant comparison here, elevated stomatal densities translated into plants with lower relative growth rates both aboveground and belowground,. Through a reduction in biomass plants, compensated for their higher water loss per unit area per se also by reducing leaf area as can be seen in the analysis of leaf area (Figure 4. 5). The typical isohydric plant response to low relative humidity involves stomatal closure keeping internal water status constant resulting in carbon starvation, followed by long-term responses such as decreases in leaf areas or adjustment of aboveground to belowground ratios. Previous studies with *A. thaliana*, reveals a lack of response in stomatal conductance to changes in leaf hydraulic conductance (K_{leaf}) (Levin *et al.* 2007) indicating either an anisohydric (Bunce 2006) or hydrodynamic (Franks, Drake & Froend 2007) nature of this plant. Anisohydric plants typically exhibit less stomatal sensitivity to evaporative demand and soil moisture, allowing large fluctuations in leaf water status (Ψ_{leaf}) The results reported here suggest that *A. thaliana* follows an anisohydric behaviour and regulates its leaf water potential by means of differential growth rates and resource allocation patterns.

From exploration of the existing data, and in accordance with its previous use in the literature (Van Der Kooij & De Kok 1996; Li, Suzuki & Hara 1998; Meyer *et al.* 2004), the use of a linear function to describe the relative growth rates of *A. thaliana*, proved to be the most adequate. Growth rates of aboveground biomass were higher under well-water conditions than under watered-limited conditions. This is to be expected as reduction in leaf area is a common response to water stress in order to reduce water loss through evapotranspiration from the leaves (Larcher 1995). Since stomatal conductance was higher and water use efficiency was lower in *sdd1-2* plants, it was expected that the stomatal density mutant would show a reduced growth rate compared to *col-5*. However, under experimental conditions the wild-type did produce 0.012 $mg^{-1}mg^{-1}day^{-1}$ less dry biomass than the *sdd1-2* mutant but this difference was not statistically significant. Further replicate observations would improve the precision of the experiment. Under water-limited conditions *sdd1-2* grew at a rate 0.002 $mg^{-1}mg^{-1}day^{-1}$ slower than *col-5*, difference but again this was not significantly different, such that both genotypes reduced their growth at the same rate.

Van den Honert (1948) proposed an analogy of Ohm's law for water transport in plants, regarding transport as a catenary process by which as the leaf loses water due to transpiration, water moves up the plant and from soil driven by physical forces as if the water column is acting as a continuous 'rope of water'. The difference between water uptake by the roots and transpirational water loss by evaporation is known as the water balance of the plant. Water balance becomes negative, as the uptake of water is insufficient to compensate for the amount of water lost through transpiration. Pressure gradients required for water movement through the xylem result from the positive water pressure generated at the roots via water uptake or negative pressures produced at the leaf level. Water in the leaves develops tension ($\Psi<0$), which is responsible for pulling water through the xylem. This is known as the cohesion-tension theory. This process is more complex that a simple Ohm's law analogy (Tyree 2007) but it has been observed that partial defoliation produces the opposite effect to make up for the loss of leaf area (Meinzer & Grantz 1990) agreeing with the observations of lower relative growth rates and reduced leaf areas in *sdd1-2*. The results from the studies here show that under well-watered conditions, *sdd1-2* exhibited higher belowground growth rates than *col-5* whereas aboveground growth rates of *sdd1-2* were not statistically different from the wild-type. This implies that CO_2 fixation is similar to an *A. thaliana* plant with lesser stomatal numbers despite different water use efficiency. Thus in order to keep carbon fixation constant, *sdd1-2* was losing more water than *col-5* in the exchange process. For this reason root growth was enhanced to a higher extent in *sdd1-2* compared to *col-5*.

Analysis of time of flowering for several plant species has been commonly explored as the averaged date of first flower (Jonas & Geber 1999; Montague, Barrett & Eckert 2008) or even as the date of first flowering in a plant population (Fitter & Fitter 2002). Hence with these methods it is only possible to assess the span of time during which flowering occurs. Other approaches include recording the date of first and last flower within a given population (Sandring *et al.* 2007) in a wild relative of *A. thaliana*, *Arabidopsis lyrata*. This measure does account for the period of time in which flower occurs but does not account for its rate. Finally, the study of the relationship between the cumulative percentages of plant reaching the flowering stage against time has been used as well (Helenurm & Barrett 1987; Gutterman & Boeken 1988; Obeso 1993) but such

data are rarely statistically analyzed. In the case of *A. thaliana*, flowering date is particularly well studied since several genes affecting time of flowering have been identified (Koornneef, Hanhart & Veen 1991; Coupland 1995); and methods to study flowering time are similar to those described for other species including: i) study of average time of first flower appearance (Lagercrantz *et al.* 1996; Callahan & Pigliucci 2002; Stinchcombe, Dorn & Schmitt 2004a); ii) analysis of observed frequencies of flowering during the flowering period (Kuittinen, Sillanpää & Savolainen 1997; Hagenblad *et al.* 2004; Werner *et al.* 2005) and iii) as cumulative percentage of plants flowering against time again, with no detailed analysis.

In this work isolated *A. thaliana* plants in the absence of stress flowered at a constant rate of about 5.5 flowers per day throughout the flowering period in both genotypes, indicating that the rate of flowering is constant until the cumulative percentage of plants reaches 100% flowering. On average *col-5* genotypes flowered approximately one day later than *sdd1-2* in the absence of water stress. It has been suggested that early flowering in *A. thaliana* is a response to an uncertain environment characterized by short seasons while late flowering would be advantageous when the environment is less disturbed and presents longer seasons (Westerman & Lawrence 1970; Jones 1971b). In this study, alteration in the watering regime alone under constant light regimes resulted in a substantial alteration to flowering. In other words, the stress of a changed water regime accelerated the transition to the reproductive stage; not only by accelerating the mean flowering time for both genotypes by two days but also by a subsequent more rapid increase in flowering rates (Fig 4. 6.B). Wild-type *col-5* under LW reached this flowering peak earlier than *sdd1-2* with a subsequent diminunition in flowering. In the case of *sdd1-2* the pattern was noticeably different; after reaching a flowering peak (18 plant flowering per day, and twice as large as *col-5* (9 plants flowering per day)) and it decreased much more abruptly. Water limitation accelerated flowering by an average of two days in both genotypes. Earlier flowering under water-limited conditions as the wild-type did not vary the timing of flowering as water availability became compromised.

Aboveground biomass at time of flowering for did not differ for *sdd1-2* and *sdd1-1* from their respective background lines under well-watered conditions. Water

limitation resulted in reduced aboveground biomass for *sdd1-2*, *sdd1-1* and their respective wild-types; the experimental frequencies of water limitation did not result in a reduced aboveground limitation of *C24* but the effect of this water-limitation did reduce aboveground biomass for *sdd1-1* at the time of flowering (Figure 4. 4.B). Aboveground response for these different mutations followed to a higher or lower extent the same trend as the growth analysis and the degree of the response probably had to do to their different sensitivity to the water regime imposed since they all had different background lines and they may not have responded to water stress to the same degree. Another thing to bear in mind is that *sdd1-1* and *tmm1-1* were not assessed for any effect that the mutation might have had on their photo-biochemistry.

Chapter 5. Genotypic responses to density in *Arabidopsis thaliana* in relation to water regimes

5. 1. Introduction

The physiological and developmental characteristics of plants growing alone in the absence of competition, as described in the previous Chapters, are basic to understanding how plants may respond to the conditions imposed by the interactions with other plants in a population. Competition is an important selective force for all types of organisms (Darwin 1858) and it can be defined as a reciprocal negative interaction among individuals (Connell 1990). Its importance as an ecological factor increases under conditions where one or more resources are limiting productivity (Fowler 1986). Individuals of the same species have similar requirements for a limiting resource to obtain optimal growth and ultimately maximize their reproductive output and they will consume this limiting resource up to a point in which the combined demand of the individuals of a population exceeds the supply.

As plants in a population develop and increase in size, the average biomass produced by an individual plant becomes limited by the availability of resources (Harper 1967) as neighbouring plants interfere with the uptake of the limiting resource in a more intense manner as the population density increases (Hara 1984). In an even-aged monospecific stand, density-dependent reductions in growth compensates for variations in density leading to a constant yield per unit area (Watkinson 1980). The fecundity of an individual plant itself is typically related to the size of the plant, which at the same time is governed by the density of the population, a reflection of the ecological success of the species in a particular habitat.

The effects of competition on plant growth and fitness varies amongst species and is dependent on physical conditions (Aerts 1999). In the case of competition for water, individuals are affected by depletion in the level of a resource by their neighbours, which strongly reciprocally limits their growth because water is a fundamental requirement for growth.

With respect to plant competition it is important to distinguish between the 'competitive effect' and the 'competitive response' (Goldberg & Werner 1983;

Goldberg & Fleetwood 1987; Goldberg & Landa 1991). Competitive effect is the ability of an organism to reduce the performance of other organisms. Competitive response is the ability to continue to perform more or less well in the presence of competitors. These two aspects of competition may be, but are not necessarily related, since the traits that provide plants with a differential competitive effect may not imply a different competitive response and vice versa (Goldberg & Fleetwood 1987). Whilst commonly considered for inter-specific interactions, these ideas have equal applicability to inter-genotypic interactions.

Studies of competitive effect have received much attention in the literature and it seems to be manifested mainly as an asymmetric process in which larger plants acquire a disproportionate amount of resources (Keddy, Fraser & Wisheu 1998). However, competitive responses are less understood (Goldberg & Landa 1991; Goldber 1996) and three strategies have been described on the basis of different trade-offs between resource use and conservation (Keddy, Fraser & Wisheu 1998): species may avoid being suppressed by acquiring resources at a higher rate, shifting resource acquisition site or time relative to neighbours, or conserving scarce resources. Unlike competitive responses, competitive effects seem to remain constant across habitats varying in productivity (Goldber 1996; Keddy, Fraser & Wisheu 1998). This means that since plant interactions vary depending on the resource availability (Pugnaire & Luque 2001), the study of the outcomes of competitive response becomes of great importance with the predictions of changing environments as a result of climate change in relation to water availability.

The study of intraspecific competition in *Arabidopsis thaliana* is of importance because of its relatively poor seed dispersal and synchronous seed germination (Baskin & Baskin 1983). However, the study of yield responses to density in *A. thaliana* is rare despite its wide use for research purposes and the opportunity that the availability of ecotypes and mutants offers to address ecological questions. The few available published studies on the subject do not assess the effects of density on biomass production under high and low resource environments as is often done in ecological research (Goldberg & Barton 1992; Goldberg & Novoplansky 1997). Ballaré and Scopel (1997) studied yield in response to density with photoreceptor mutants of *A. thaliana*;

but they did not model yield density relationships in specific resource-limited conditions such as water stress. Previous research efforts have compared two *A. thaliana* genotypes differing in dhurrin production in the leaves (Damgaard & Borksted 2004), but this study did not compare the density response under different abiotic conditions i.e. a resource gradient; moreover the biomass of isolated plants was not considered in the evaluation of crowding. Later on, *A. thaliana* was used for the study of the responses of vegetative and reproductive yield in response to intraspecific density using two stomatal density mutants; *sdd1-2* and *tmm1-1* (Alwerdt *et al.* 2006) which did not use any empirical model for population growth and did not compare this density response to a resource limited condition that may alter this relation to intra-specific relations. Moreover, this study used *col-0* as *sdd1-2* and *tmm1-1* mutants background line; while the correct wild-types are *col-5* for *sdd1-2* and *col-gl1* for *tmm1-1*. Alwerdt et al (2006) found that there was a difference between both mutants and *col-0* in their response to plant density although this results should be taken with caution for the mentioned reasons.

Two approaches were used to examine yield responses to density. The first study (Experiment 1) considered the intra-genotypic response in vegetative and reproductive biomass to density in the *col-0* ecotype. Redei selected this original Columbia ecotype from the non-irradiated Laibach Landsberg population, as it was a particularly fertile and vigorous genotype that responded well to changes in photoperiod (Redei 1975). This ecotype is the most commonly studied ecotype in *A. thaliana* and from which *sdd1-2* is derived through its background line *col-5*, a natural mutation of *col-0*. The second study (Experiment 2) compared intra- and inter-genotypic responses to density using an additive series model. In both experiments, genotypic responses were examined in relation to LW and HW watering regimes, as described in the previous Chapter.

5. 2. Materials and methods

5. 2. 1. Experiment 1

Growth conditions

Arabidopsis thaliana plants, *col-0* ecotype, obtained from the European Arabidopsis Stock Centre, NASC (http://arabidopsis.info), were grown in a plant growth chamber planted in 230 x 176 x 55 mm flats with a mixture (3:1) of compost and sand. The chamber was maintained at 21˚C during the day and 19˚C during the night with 50% relative humidity and light intensity in the range of 150 and 200 μmol m^{-2} s^{-1} PPFD. Two water regimes were applied as treatments. Initially plants in both were watered as described previously (Chapter 4). Twenty days after emergence, high water treatments (HW) were watered to soil capacity on a weekly basis, while low water treatments (LW) were watered to soil capacity on a bi-weekly basis.

Seeds were pipetted individually from a 0.5% agar suspension were planted in a lattice grid in a range of densities. Densities varied from plants growing alone (30.7 plants m^{-2}) to 15, 24, 70, 80, 177 and 176 plants per flat (460, 110, 2150, 2703, 4310 and 5898 plants m^{-2}) seeds being placed at the same distance from each other vertically and horizontally (4, 3, 2, 1.75, 1.5 and 1.25 cm from each other). Each density was replicated four times excepting for the highest density treatment which was replicated twice and the lower density of 460 plants m^{-2} which was replicated five times. Within each replicate, the time of flowering of every individual plant (phenotypic growth stage 6.00 where the first flower is visible was recorded on a daily basis using the individual coordinates of each plant. A destructive harvest was conducted 15 days after plants entered the flowering stage (DAF). Fitness was recorded as measurements of vegetative and reproductive dry biomass by placing the separated vegetative and reproductive portions of the plant in an oven and dried at 60°C for 48 h. Overall 299 vegetative and 306 reproductive measures of dry biomass were taken under well-watered conditions (HW); and 288 vegetative and 290 reproductive measures taken under water limited conditions (LW).

5. 2. 2. Experiment 2

Seeds of the two genotypes *sdd1-2* and *col-5* were sown into flats from a 0.5% agar suspension as described above that allowed a) examination of intra-genotypic competition over a density range of 1 – 88 plants per flat, and b) response at a constant target density to increasing densities of neighbours from 1 – 44 plants per flat. Figure 5. 1 indicates diagrammatically the genotype mixtures and combinations that were used in the experiment. The first row and column of the set of combinations represent mono-cultures of each genotype over a density range up to 88 plants/flat. The second row and column (initiated with a density of three plants) represents a paired additive series of densities enabling the effect of inter-genotypic competition to be assessed relative to intra-genotypic genotypic, over the same density range. Since separate harvests of each genotype were taken from each pair of replicates, statistical independence was acquired. If genotype 1 is the target, then the second row in the table represents increasing density of genotype 1 (intra-genotypic competition), in the consistent presence of 3 plants of genotype, whereas the second column represents the effect of increasing density of genotype 2 (inter-genotypic competition).

Plants were grown in a growth chamber planted individually in 230 x 176 x 55 mm flats containing a mixture (3:1) of John Innes #3 and sand. The chamber was maintained at 21°C during the day and 19°C during the night with 50% relative humidity and light intensity in the range of 150 and 200 $\mu mol\ m^{-2}\ s^{-1}$ PPFD. Two watering regimes, high water (HW) and low water (LW) were implemented where soil was watered to field capacity from underneath at different frequencies. HW treatment was kept well watered during the whole length of the experiment. For LW treatment, plants were kept well watered during the first two weeks after emergence, then watering ceased for 14 days, and were then watered again 14 days later (40 days after emergence). Plants were watered with the same frequency as HW treatment after 40 DAE.

I assigned random coordinates using a grid as a guide to sow individual seeds within the pot, allowing the location of plants at the time of harvest. This was critical for mixture plantings. The experiment was then divided into two blocks planted with a week's difference. Each block was planted within two days in order to stagger harvests.

Each one of the 16 planting combinations was explored under two water

regimes (HW and LW), duplicating the amount of pots in order to carry independent observations (so I only observed the performance of one genotype per pot). Every treatment was replicated three times to make a total of 192 pots used in this experiment.

Plants were destructively harvested at and dried to constant weight as described above.

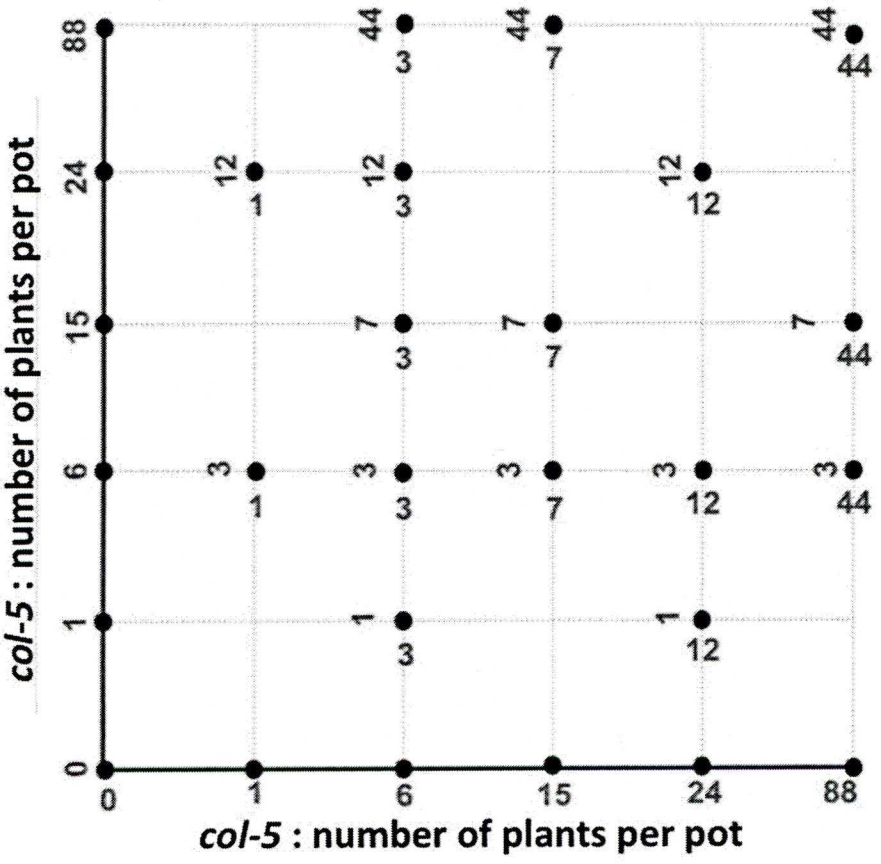

Figure 5. 1. Genotype combinations used in experiment 2.

Data analysis

Experiment 1

Vegetative to reproductive allometric relationship

The best-fit equation to express the relationship between the vegetative and reproductive biomass 15 days after flowering (DAF) under well watered conditions was determined using TableCurve 5.01 (Systat Software Co., Point Richmond, CA) as :

$$y = a + b\,(x) \qquad (1)$$

where y is the natural logarithm of reproductive biomass 15 DAF, x is the natural logarithm of vegetative biomass 15 DAF, b is the slope, and a is the y-intercept. Analysis of covariance (ANCOVA) was used to compare linear responses using the statistic program package R (http://cran.r-project.org/); modeling reproductive biomass (the response variable) as a function of water regime and vegetative biomass. Water regime was a factor with two levels (HW and LW) and vegetative biomass is a continuous variable. The model therefore has four parameters: two slopes (one slope for HW and another slope LW) and two intercepts (one for HW and another LW):

Yield-density analysis

The relationship between plant yield and population density has long been appreciated as important from both a theoretical and practical point of view (Bleasdale & Nelder 1960; Willey & Heath 1969; Hassell 1975; Watkinson 1980; Damgaard 2008; Goldberg *et al.* 2008; Nelder 2009; Sharpe & Dent 2009). Willey and Heath (1969) finally proposed the use of inverse relationships between yield and density as originally proposed by Bleasdale and Nelder 1960 and further revised by Watkinson (1980).

This relationship between plant performance and density in a monoculture has been commonly described in the literature by the following equation:

$$w = w_m(1 + aN)^{-b} \qquad (2)$$

where w is the mean plant biomass, N is plant density, W_m is either vegetative or reproductive mean biomass of a plant growing in the absense of competition; the reciprocal of a is an estimate of the space required for one isolated plant to grow to

maximum size and, b is a measure of the rate at which competition decays as a function of density (Watkinson 1980; Vandermeer 1984). Where $b = 1$ there is exact yield compensation for density, over-compensation when $b < 1$ and under-compensation when $b > 1$.

Experiment 2

A linear fixed effects model (REML) was used to analyse the response of *col-5* and *sdd1-2* separately to watering regime, density and 'competitor' (intra- versus inter-genotypic). The term 'competitor' in the table compares the response to of the target genotype to increases in its own density with the response to increases in density of the alternative genotype. Data were log transformed.

5. 3. Results

5. 3. 1. Experiment 1

Allometric relationships

Despite significant variation amongst individual plants there was a significant linear relationship between vegetative and reproductive biomass in both water regimes (Figure 5. 2, Table 5. 1).

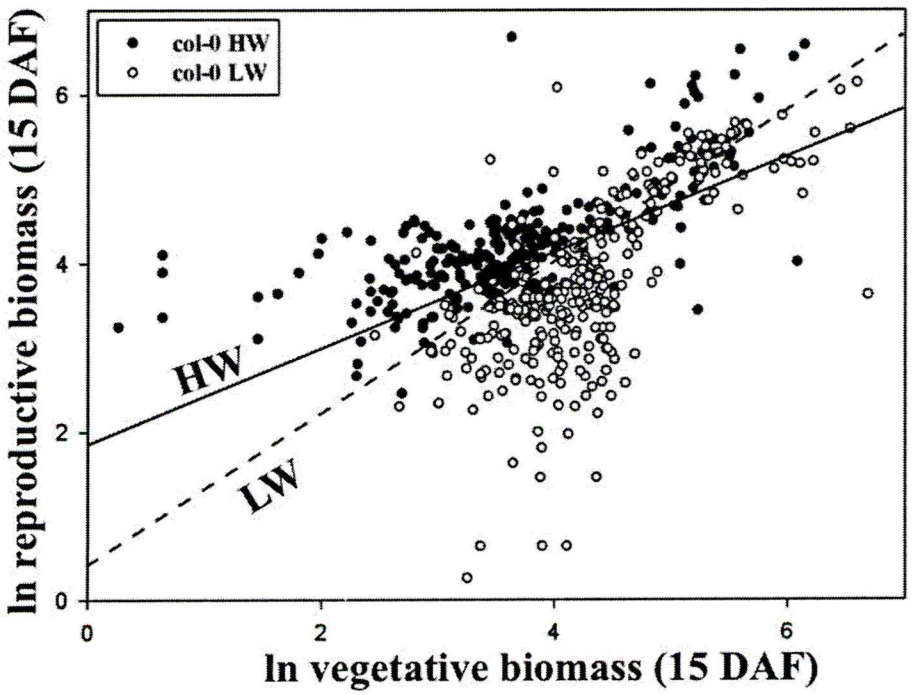

Figure 5. 2. Allometic relations between vegetative and reproductive biomass 15 days after plants entered the flowering stage under well watered (HW; continuous line) and water-limited (LW; continuous line) conditions.

Change in water regime affected the allometric relationship between vegetative and reproductive biomass and there was a simple increase in reproductive biomass per unit vegetative biomass under HW (slopes and intercepts significantly differed between water treatments (Figure 5. 1, Table 5. 1, Table 5. 2). The increased slope and higher intercept observed under LW suggest that in *col-0,* for a given vegetative biomass

reduced water availability increases the ratio between vegetative to reproductive yield. This relation tends to approach vegetative to reproductive ratios observed for well-watered plants as vegetative biomass increases

Table 5. 1. The allometric relation between vegetative and reproductive biomass at 15 days after flowering according to eq. 1.

Treatment	*a*	*b*	Model fit standard error	F	P value	n
***col-5* HW**	1.937	0.525	0.783	103.708	<0.01	299
***col-5* LW**	0.241	0.864	0.611	388.593	<0.01	283

Table 5. 2. ANCOVA analysis of the effect of water regime on the allometric relation between vegetative and reproductive biomass of *col-0*.

Source of variation	df	SS	F value	P
Water regime	1	704	340.09	<0.01
Vegetative biomass	1	84.95	451.15	<0.01
Water regime x vegetative biomass	1	64.35	341.53	<0.01
Residuals	577	108.64		

Yield density relationships

The yield of both vegetative and reproductive biomass of *col-0* declined with increasing density and no mortality of plants was observed over this density range (Figure 5. 3 and 5. 4). The yields of isolated plants (w_m) were similar under both watering regimes with respect to vegetative biomass (Table 5. 3) but reproductive biomass was higher under the HW, even though the estimate had a much larger standard error (Table 5. 4).

The reciprocal of parameter *a*, (an estimate of the area needed to achieve w_m) was larger under HW than LW for vegetative biomass, and this relationship was reversed for reproductive biomass. Whereas *b* was not significantly different from unity under LW, it was significantly greater than 1 in the vegetative response. For reproductive biomass, parameter values were less than 1.

The results from this experiment suggest for this ecotype yield responds strongly to density over the range 1 – 80 plants per flat, equivalent to 31 – 2700 plants m^{-2}. Whilst vegetative and reproductive biomass showed a similar response, the model

suggests that the yield of reproductive biomass in HW is consistently elevated over that in LW over the entire density range in contrast to vegetative biomass.

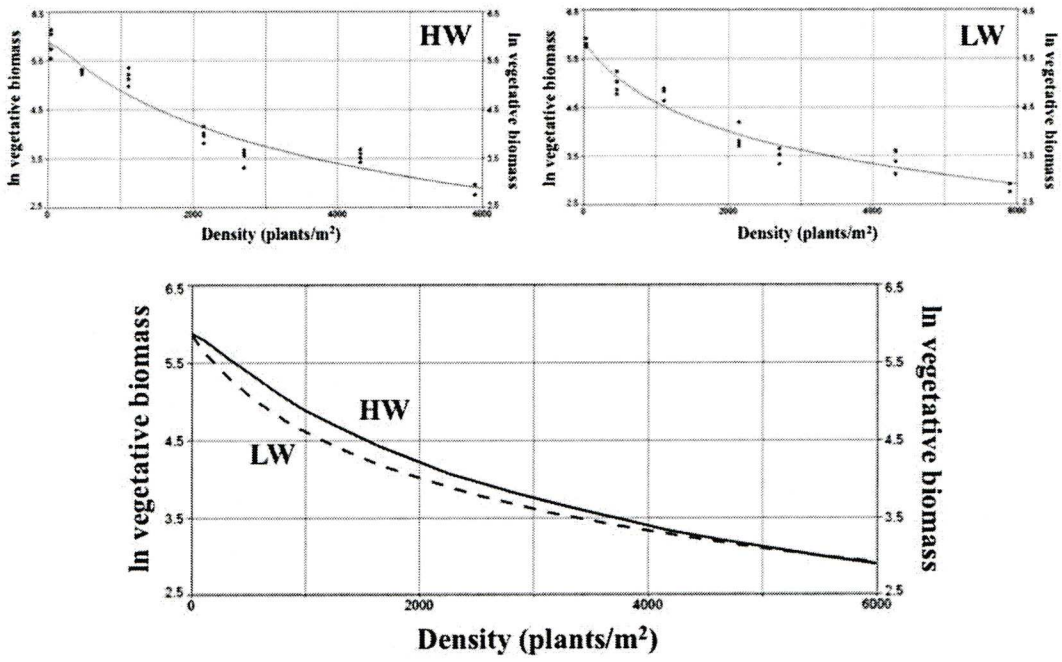

Figure 5. 3. Vegetative individual biomass in relation to density in *col-0* under well watered (HW; straight line) and water-limited conditions (LW; dashed line) log plots, fitted curves follow eq. 2. The top figures show the data separately including the data points; whilst the figure below presents the model fits for comparative purposes.

Table 5. 3. Curve fit and parameters estimate for vegetative biomass; eq. 2.

Treatment	W_{max}	SE	a	SE	b	SE	Fit SE	r^2	P
***col-0* HW**	354.83	49.653	0.0015	<0.001	1.35	0.141	0.281	0.929	<0.01
***col-0* LW**	342.732	45.717	0.0022	0.002	1.101	0.101	0.281	0.929	<0.01

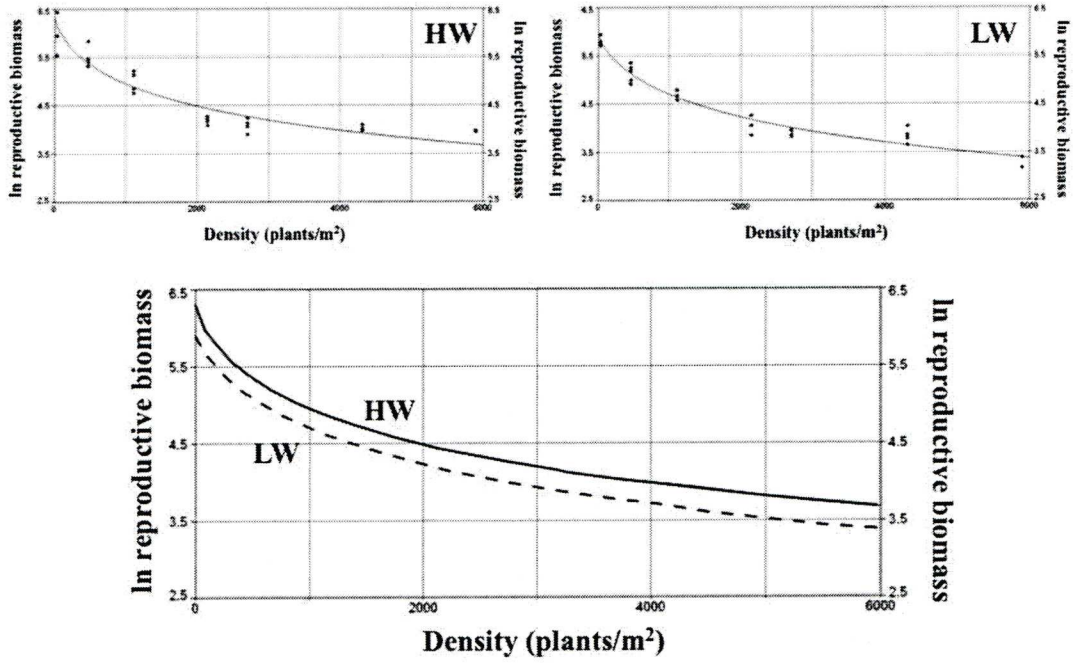

Figure 5. 4. Reproductive individual biomass in relation to density in *col-0* under well watered (HW; straight line) and water-limited conditions (LW; dashed line) log plots, fitted curves follow eq. 2. The top figures show the data separately including the data points; whilst the figure below presents the model fits for comparative purposes.

Table 5. 4. Curve fit and parameters estimate for reproductive biomass; eq. 2.

Treatment	W_{max}	SE	a	SE	b	SE	Fit SE	r^2	P
col-0 **HW**	558.4	115.282	0.0037	0.002	0.834	0.114	0.27	0.91	<0.01
col-0 **LW**	370.834	42.533	0.0027	0.002	0.888	0.076	0.169	0.968	<0.001

5. 3. 2. Experiment 2

Figure 5. 5 illustrates boxplots of the response (biomass per plant) of each genotype to increasing density in monoculture in relation to the two watering regimes. Median yields showed a noticeable monotonic decline with density up to 24 plants per flat in both watering regimes, but the reduction in the size of plants with a further approximate four fold increase in density (88 plants / flat) was not as marked as seen over the lower density range. Conspicuously there was considerable variation in individual plant size at densities greater than 3 per flat.

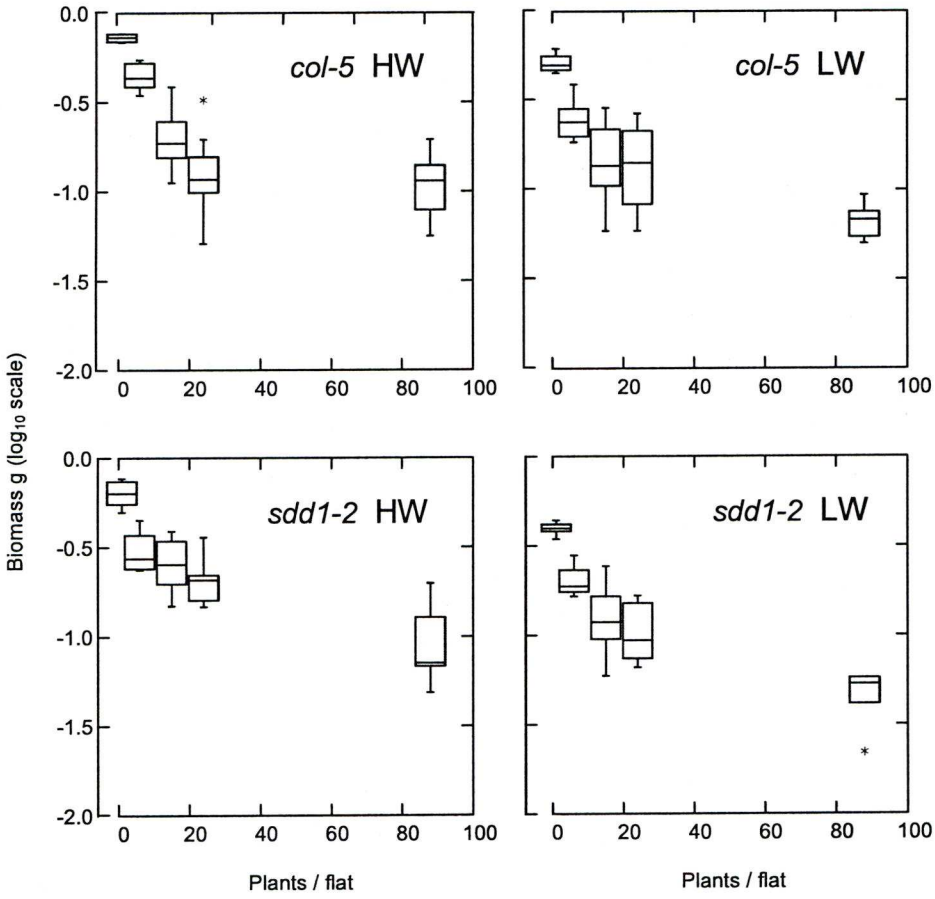

Figure 5. 5. Box plots of the vegetative biomass of *col-5* and *sdd1-2* in relation to planting density at LW and HW. Biomass per plant is expressed on a $\log_{10}$ scale.

Tables 5. 5 and 5. 6 present the analyses of the linear fixed effects model for the two genotypes in relation to watering regime and competitor. For both genotypes, watering regime had a significant effect on vegetative biomass, as did density and the responses to density in relation to watering regime. The comparison of the response to increasing intra-genotypic competition as opposed to inter-genotypic competition at a target density of three plants / flat, averaged over watering regimes was not significant in *col-5* whereas the response was significant for *sdd1-2*. In Figure 5. 6 for *col-5* it can be seen that the linear responses to density (*col-5* or *sdd1-2*) are very similar at HW with a smaller discernable difference under LW. Contrastingly for *sdd1-2* (Figure 5. 7), there was a statistically significant third order interaction, reflected in the different slopes of the density responses at both watering regimes and in response to competitor. In this

mutant the response to inter-genotypic competition was greater than the response to intra-genotypic competition at HW, whereas at LW the situation was reversed. As Figures 5. 6 and 5. 7 show at LW in particular, yield declines in response to density were more variable than those seen at HW.

Table 5. 5. Linear fixed effects model (REML) analysis of the response of *col-5* to watering regime, density and 'competitor' (intra- versus inter-genotypic).

Term	*Wald statistic*	*df*	*Wald/df*	Chi-sq prob
Watering regime	29.41	1	29.41	<0.001
Competitor	0.83	1	0.83	0.362
Density	130.22	4	32.55	<0.001
Watering regime x competitor	0.06	1	0.06	0.809
Watering regime x Density	29.97	4	7.49	<0.001
Competitor x Density	3.11	4	0.78	0.539
Watering regime x Competition x Density	2.20	4	0.55	0.699
Residual	Estimate	SE		
	0.000964	0.0001437		

Table 5. 6. Linear fixed effects model (REML) analysis of the response of *sdd1-2* to watering regime, density and 'competitor' (intra- versus inter-genotypic).

Term	*Wald statistic*	*df*	*Wald/df*	Chi-sq prob
Watering regime	50.78	1	50.78	<0.001
Competitor	4.32	1	4.32	0.038
Density	102.23	4	25.56	<0.001
Watering regime x competitor	5.88	1	5.88	0.015
Watering regime x Density	17.23	4	4.31	0.002
Competitor x Density	5.37	4	1.34	0.252
Watering regime x Competition x Density	50.78	1	5.78	<0.001
Residual	Estimate	SE		
	0.000823	0.0001294		

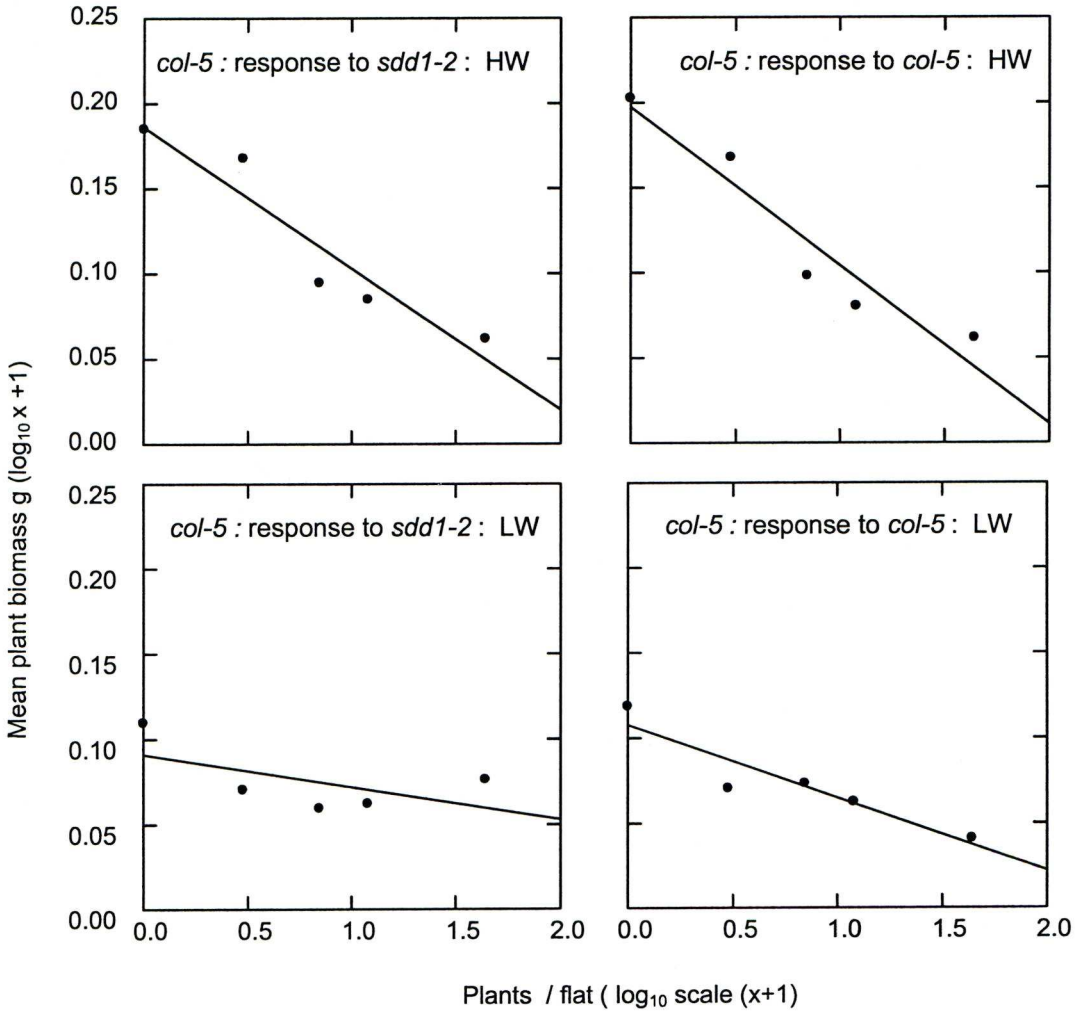

Figure 5. 6. Linear relation between plant density and plant biomass for *col-5* in intra- and inter-genotypic competition under well-watered (HW) and water-limited conditions (LW).

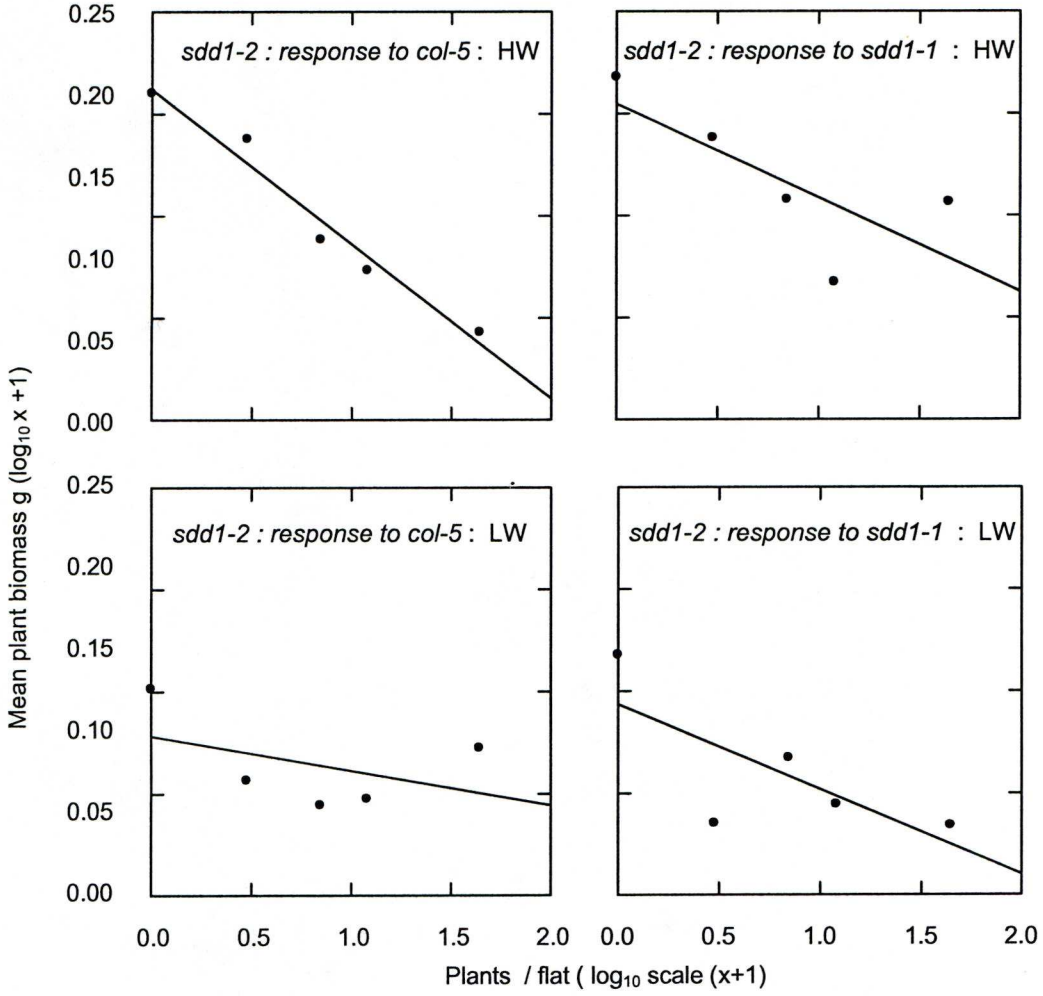

Figure 5. 7. Linear relation between plant density and plant biomass for *sdd1-2* in intra- and inter-genotypic competition under well-watered (HW) and water-limited conditions (LW).

5. 4. Discussion

The life history of *A. thaliana* involves a phenological switch with the transition from solely vegetative growth to reproductive growth (bolts) and subsequent expression of flowers and seed. Figure 5. 2 for *col-0* shows that under well-watered conditions the relationship between vegetative and reproductive biomass at 15 DAF was a linear one over at least 5 orders of magnitude (natural logarithmic scale). In contrast the data describing the allometric relationship between vegetative and reproductive biomass at LW, whilst showing a linear trend, was much more variable. These data were derived from individual plants grown in populations over a range of densities in which size hierarchies were present. Whilst the effect of the LW regime was in general to reduce the size range of variation in vegetative biomass, the variability in reproductive biomass at 15 DAF was much greater and may be a consequence of the position of individual plants within the size hierarchy and time of initiation of flowering.

The experiments on yield density relationships in *col-0* examined mean performance of plants in response to a wide range of densities and clearly illustrate a monotonic decline in mean yield with plants under LW exhibiting lower biomass than under HW in both vegetative and reproductive biomass, as is to be expected given resource limitation. Whilst the goodness of fit of the overall model was high for each data set (Tables 5. 3 and 5. 4) and in all but one case (w_m) parameters were estimated with reasonable precision as judged by their standard errors, estimates of *a* were strongly dependent upon the first two densities employed in the experiment and the yield responses did not reflect a gradual increase in the intensity of competition at low densities. Greater confidence can be place in estimates of *b* and it is interesting that estimates of this parameter were less than unity for reproductive biomass. In the flowering stage, the rate of vegetative growth significantly diminished and yield density relationships will reflect resource partitioning that is more dependent on prior acquired biomass than on growth continuing through the bolting and flowering stage. The estimates of *b* for vegetative biomass at HW suggest that over-compensation for density was occurring. Smaller plants than might be expected to occur under the assumption of a constant final yield at high density may have resulted from the absence of density

dependent mortality, although such responses are more typical of reproductive yield components than vegetative ones (Silvertown & Charlesworth 2001)

The use of two additive series responses to density (experiment 2) represent 'slices' of a full response surface analysis (Law & Watkinson 1987; Bullock, Mortimer & Begon 1994; Inouye 2001) that have rarely been completed because of the high experimental demands required to assess responses in two species (genotype) space, with statistical independence of observations of individual species. In experiment 2, a full response surface analysis over a range of densities and mixtures was not completed due not only to the experimental size required but also due to the need to identify different genotypes in mixture. The variability in yield amongst individuals already remarked upon earlier, was present in this experiment as evidenced in Figure 5. 5, and in contrast to the density response of *col-5*, the mean plant biomass at high density (88 plants / flat) was not as pronounced in comparison to yields at lower density. For this reason fitting equation 2 was not considered an appropriate form of analysis and a REML analysis used instead. Figures 5. 6 and 5. 7 illustrate the intra- and inter-genotypic responses and impose a linear decline curve on yield by linear regression. As observed earlier these linear relations were much more consistent at HW than LW. At LW, discounting the yield at the lowest density, biomass per plant was smaller and the response to density reduced. The interpretation of intra- / inter-genotypic effects is then contingent on estimation of yield at low densities. With this caveat and the fact that the analysis is focused on the target population of 3 plants/flat, the *col-5* genotype showed no difference in response to density, either mono-genotypic or inter-genotypic at HW. For the *sdd1-2* this was not the case with inter-genotypic competition greater than intra-genotypic competition and the reverse at LW. These results suggest that there may be fitness tradeoffs as a result of the *sdd1-2* mutation. Further experimentation is required to support this suggestion including analysis of rooting depth, in relation to density and biomass partitioning between roots and shoots in density induced size hierarchies.

But intuitively from the observation of Figure 1, from what happens to *col-5* and *sdd1-2* in Chapter 4 and as we have seen from the analysis of density response (described below); most of the data for the low water treatment is driven by what happens at lower vegetative biomass since water affects growth by reducing rosette size.

The study of the effect of plant densities on plant growth is important to determine the dynamic behaviour of the plant in a community. The effects of competition on plant fitness varies among species and is dependent on physical conditions (Aerts 1999). The intensity and reciprocity of competition among plants not only depends upon external influences such as resource availability or plant density (Wilson & Tilman 1993; Dyer & Rice 1999; Grime 2001). According to the isocline approach to resource competition, equilibrium requirements are given by the resource-dependent growth isoclines of the species (Tilman 1980; Tilman 1982).

Two major physiological trade-offs have been argued to drive competition under different environmental conditions: i) trade-off between obtaining high relative growth rates (RGR's) and maximize resource adquisition vs. resource conservation by reducing biomass turnover (Grime 1977) and ii) trade-off between allocation of the carbon gained through photosynthesis to root growth in order to acquire belowground resources vs. allocation to aboveground growth to capture aboveground resources (Tilman 1988).

Competitive interactions among plants are generally asymmetric, so large individuals will uptake a disproportionate amount of resources relatively to small individuals (Weiner & Thomas 1992; Thomas & Bazzaz 1993; Weiner *et al.* 2001). According to this, I expected before this study, that when plants compete for an aboveground resource as in well watered conditions where plants grow to bigger vegetative biomass and so larger individuals will shade smaller individuals as they compete for light, over-competing their smaller neighbours while keeping belowground biomass more symmetric among individuals since they are not competing for belowground resources. We would expect the opposite effect in the water-limited treatments; plants compete for a belowground resource so individuals reach a higher asymmetrical growth where plant with a larger root biomass will uptake more water and therefore uptake more water while keeping rosette growth more symmetric since plants will overlap each other depleting light from neighbours at higher densities than in the case of well-watered treatments.

However, from our results we can conclude that plants growing in the abscense of competition, grew larger when well watered as we would intuitively expect and as we have seen for *col-5* and *sdd1-2* in Chapter 4: the more available resources, the larger

plants grow. But when we look at the relation between vegetative growth and plant density seems that the obtained measure of competition suggest that its intensity and range of densities at which is more intense, is higher under well-watered conditions. This contradicts my previous expectations; but a possible explanation for this could be that water deficit has been reported to enhance root growth in *A. thaliana* (Van Der Weele *et al.* 2000) but also decreases leaf areas to avoid loss of water through evapo-transpiration (Larcher 1995; Taiz & Zeiger 2006) resulting in reduced aboveground to belowground ratios. This resource allocation for growth not only to uptake more of the limiting resource but to reduce the loss of the same resource affects the measure of competition obtained through the density response curves: this measure of competition include direct competition (plants shading each other competing for light) and interference competition brought to the aboveground to belowground level in which intense competition for a belowground resource affects more rosette growth than when competing for an aboveground resource.

As to why this relation in inverted when we look at the density-yield and reproductive biomass, the allometric relations identified between rosettes and reproductive biomass gave us a simple explanation. For a given amount of vegetative growth, water-limited plants produce lesser reproductive biomass especially at smaller rosette sizes. For this reason water affects reproductive yield to a higher extent.

But why is this relation lower under water-limited plants? The less water, the more limited photosynthesis is, so the plant fixes less carbon and thus growth allocated to reproductive parts of the plant is lesser.

In this experiment we did not observed density-dependant mortality. We would start to see mortality if we had kept increasing plant densities while keeping the same biomass per pot following the law of constant yield (Watkinson 1980); but I was not interested in the study of density dependant mortality.

It is important to keep in mind that the study of competitive interactions through this population dynamic model is undoubtedly simplistic in the sense that deterministic models rely on data so that the model will not be able to make predictions outside the domain given by the data and does not imply that findings here are applicable to every possible environmental condition. Nevertheless, focusing attention on a limited set of

parameters with biological meaning is of importance as it provides us with valuable information to understand the population dynamics of a rapid-cycling annual plant of major importance in biological research such as *A. thaliana.*

This is the first known attempt to simultaneously model the density response of *A. thaliana* vegetative and reproductive biomass production in relation to resource availability. The study of plant-plant interactions is important in determining the evolutionary relevance of phenotypical attributes of plants brought about from the different ecotypes and mutants available for *A. thaliana* and which are usually studied in the absence of competition.

Finally, the determination in *col-0* of the densities at which competition is more intense allowed me to choose a suitable density for the study of target competition for *sdd1-2* and *col-5* in relation to water conditions as described in the following Chapter.

The work reported in this Chapter examines the density responses (vegetative and reproductive biomass) of the two *A. thaliana* mutants in relation to the belowground resources that are mediated by water. By design, the experiments themselves shed little light on the inter-relationships between above and belowground biomass, but did highlight the extend to which differences occurred between mutant and wild-type. The considerable variation that was evident amongst plants of the same genotype under the same experimental conditions.

As discussed in the introductory Chapter, both inter- and intra-population genetic variation is well known in *A. thaliana*, but the inter-plant variation reported here is entirely environmentally induced as genotypes were supplied from a single accredited seed source and multiplied under controlled conditions. Growth variation may result from minor site variability at the time of planting and early germination initiating the development of size hierarchies that became progressively skewed with time. The extent to which this size hierarchy in aboveground biomass is mirrored in belowground biomass was not investigated and regardless of partitioning ratios, soil resource acquisition will depend upon root distribution. Plants in these experiments were watered from beneath to avoid damaging the canopy structure and displace the seeds/seedlings during the planting period. Flats themselves were 55 mm deep and roots of *A. thaliana* were observed to extend over 100 mm under well-watered conditions in the absence of

competition in preliminary trials. Differential distribution of roots in the soil profile according to time of emergence may contribute to further competitive skewing of size hierarchies.

In conclusion, the studies discussed here contribute to a further understanding of competitive interactions amongst *A. thaliana* genotypes differing in stomatal density. There are relatively few published studies on density responses in *A. thaliana* in general and those have been of limited scope. Alwerdt et al (2006) studied competition between *sdd1-2* and *tmm1-1* stomatal density mutants against *col-0* as their (erroneous) wild-type and found that the mutants had a lower threshold for responding to intra-genotypic competition relative to *col-0*. As seen in preliminary results not shown in this thesis, growth of *col-0* is greater to that of its glabrous derivate *col-5* under well-watered conditions, so the outcome of this study may be affected by the erroneous wild-type used.

A key finding of this work was the need to understand in greater detail above and belowground biomass partitioning in response to competition. This is addressed in the next Chapter.

Chapter 6. Genotypic interactions – above and belowground biomass in *col-0* and *sdd1-2*

6. 1. Introduction

Genetic variation within a native plant population is a key component of a species evolutionary potential. For example, as the size of native populations declines following competitive interactions with introduced species, loss of native genetic diversity may reshape their future evolutionary potential (Ellstrand & Elam 1993; Grant *et al.* 2003; Fridley, Grime & Bilton 2007), effectively re-ordering competitive outcomes into native gene pools. As an example, native plant communities subjected to exotic invasions undergo selection (Mealor & Hild 2006; Mealor & Hild 2007) such that surviving members of the population may possess a competitive advantage against the invader. (Ferrero-Serrano *et al.* 2008; Ferrero-Serrano, Hild & Mealor 2010).

Genetic diversity may manifest itself in subtle niche differentiation both spatially and temporally. Genotypic variation within a population may affect inter-genotypic competition so that different resources and plant attributes assume different levels of importance in different habitats (Aerts 1999). Genetic variability seems to play a role in the competitive response of plant populations to water stress (Klikoff 1966).

Studies showing the direct effects of specific genes on competition are limited (Schmitt, McCormac & Smith 1995; Andalo, Goldringer & Godelle 2001; Bates & Lynch 2001; Cahill, Kembel & Gustafson 2005; Alwerdt *et al.* 2006) and comparative mutant analysis is a useful tool to study the outcome of competitive interactions

The study of *A. thaliana* mutants is an useful tool to study the outcome of competitive processes (Cahill, Kembel & Gustafson 2005) and in this respect, the availability of *A. thaliana* mutants is of particular value given the considerable understanding of the species biology that can be employed to investigate ecological processes (Pigliucci 2002)

As discussed previously, the only study on plant competition using stomatal mutants was conducted by Alwerdt *et al.* (2006) but did not address the effects of differing abiotic conditions on the competitive process. These authors conducted an experiment assessing fitness variation in response to plant density by using the *sdd1-2* and the *tmm1-1* mutants, finding differences in growth between both mutants. In this study, the authors used the *col-0* ecotype as a wild-type instead of the Columbia-derived background lines; namely *col-5* for *sdd1-2* and *col-gl1* for *tmm1-1*. Clearly critically evaluating the impact of individual mutations on fitness requires assessment of relative performance of mutant with the relevant background wild-type.

Work presented in the previous Chapter indicated that inter- and intra-genotypic competitive effects were measurable in comparisons of *col-0* and *sdd1-2* and arguably that relative biomass partitioning to roots and shoots may play a role in governing competitive interactions, as mediated by water availability.

This Chapter presents a further analysis of inter- and intra-genotypic competitive effects on both above and belowground biomass in relation to watering regimes using the same genotype combinations as before.

6. 2. Materials and Methods

6. 2. 1. Plant materials

A. thaliana plants were grown in a growth chamber planted individually in 230 x 176 x 55 mm flats with a mixture (3:1) of compost and sand. The chamber was maintained at 21°C during the day and 19°C during the night with 50% relative humidity and light intensity in the range of 150 and 200 μmol m^{-2} s^{-1} PPFD. Water regimes were applied as described previously.

6. 2. 2. Experimental design

Seeds of each genotype were individually pipetted from a 0.5% agar solution and at an overall density of 1000 plants m^{-2} (27 plants / flat). Seeds were placed at the same distance from each other in a lattice grid following a non-random arrangement. Every flat consisted on two target plant and every flat was replicated five times harvesting two target plants for every flat. There were 16 treatments for both genotypes as a target in monoculture or mixture under well-watered or water-limited conditions.

For this study, a non-random planting design was specifically chosen for practical reasons of genotype identification. A target plant (either *col-5* or *sdd1-2*) was surrounded by either individuals of the same genotype in the case of intra-genotypic competition; or individuals of the opposite genotype in the case of inter-genotypic competition (Caton, Cope & Mortimer 2003). The use of this kind of non-random design however has consequences for the analysis of competition experiments (Mead 1967; Damgaard 2004). It has been shown theoretically that it results in a decrease in the variation in fitness components of co-specific individuals (i.e. *sdd1-2* and wild-type) (Damgaard 2004). This decrease in variation increases the chances of detecting a difference in the competitive ability amongst genotypes. This is advantageous in that it potentially maximizes the power of the experimental approach in detecting inter-genotypic differences.

6. 2. 3. Data analysis

Within each replicate, the time of flowering was recorded on a daily basis as described in Chapters 4 and 5. Flowering rate was analyzed following the methods described earlier. Regression analyses were applied to the data following inspection of the observed responses. In most instances the most parsimonious model was a linear response but in two instances a non-linear fit was applied, (Chapter 4).

Measurements of aboveground and belowground dry biomass at the time of flowering were made by weighing separated shoot and root portions of plants after oven drying at 60°C for 48 h.

Statistical analysis was conducted using the statistic program package R (http://cran.r-project.org/) by a two factor ANOVA using Type III sum- of-squares. Normality of the residuals was tested with the Shapiro–Wilk test. Homogenity of variance was tested using the Fligner-Killeen test and comparisons of means using the post-hoc Tukey pair-wise test at the $\alpha = 0.05$ level.

The Relative Neighbouring Effect (RNE) (Markham and Chanway 1996) was used to assess competitive interactions Grace (1995) argued that this index had utility for examining competition in plants.

$$RNE = (P_{-N}-P_{+N})/X \quad (1)$$

where P is the performance of plants in the presence

(P_{+N}) and absence (P_{-N}) of neighbors.

X is P_{-N} when P_{-N} is greater than P_{+N}, and

X is P_{+N} when P_{+N} is greater than P_{-N}.

This index ranges from -1 to +1 with negative values indicating facilitation and positive values indicating competition.

Due to the existence of fixed upper and lower bounds ($-1 \leq RNE \leq +1$), this index creates problems and biases where the average intensity of competition or facilitation is high and plant performance is spatially variable. As a correction an *arcsin* transformation (Oksanen, Sammul & gi 2006) was used.

6. 3. Results

6. 3. 1. Biomass

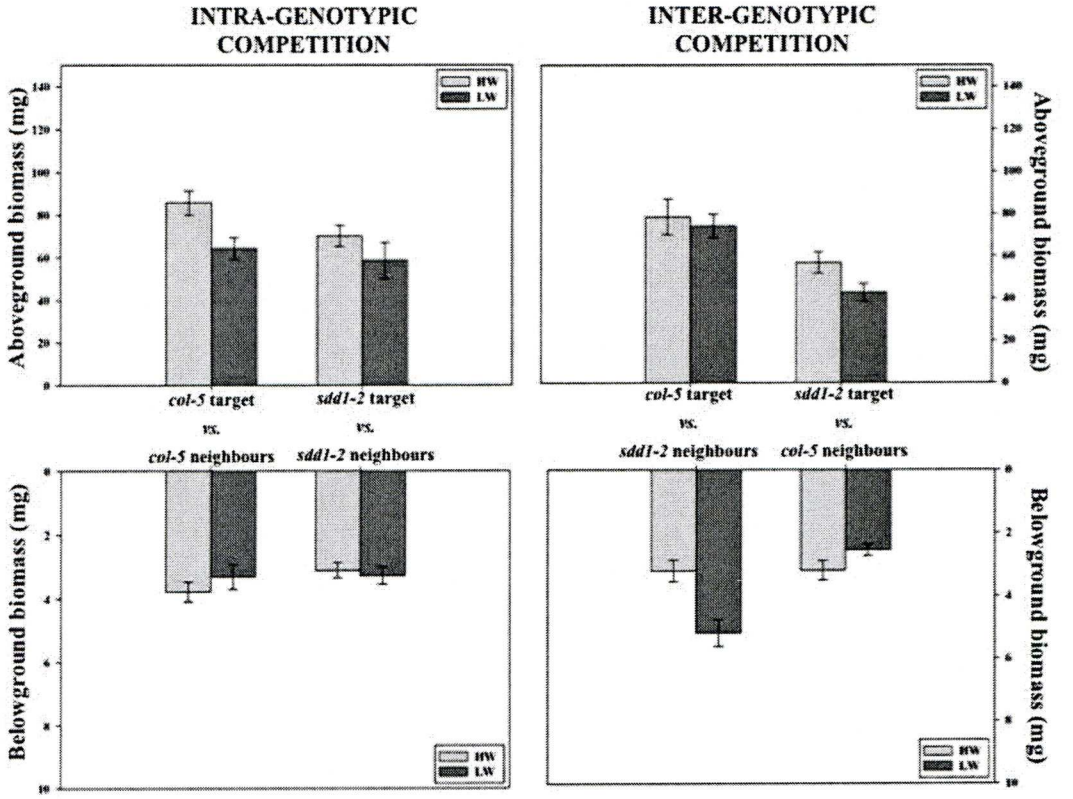

Figure 6. 1. The effects of intra- and inter-genotypic competition on above and belowground biomass under well watered (HW) and water-limited (LW) for *sdd1-2* and *col-5*. Results are mean ± s.e.m.

Figure 6. 1 displays the above and belowground biomass yields in response to intra- and inter-genotypic competition and Tables 6. 1 and 6. 2 indicate the statistical significance of individual imposed sources of variation. Aboveground under water-limited plants were statistically smaller ($P<0.05$) than under well-watered conditions when competing in monogenetic stands but not in a mixture, even though means followed the same trend. Aboveground biomass of the wild-type exceeded that of the mutant, both under intra- and inter-genotypic competition. For root biomass there were no significant effects under intra-genotypic competition in contrast to under mixture. Significant differences

in the latter were due to increased root growth of *col-5* in mixture with *sdd1-2* (Figure 6. 1).

Table 6. 1. Two-factor ANOVA for aboveground biomass at time of flowering for *col-5* and *sdd1-2* growing in intra- and inter-genotypic competition under high water (HW) and water-limited (LW) conditions. The term genotype refers to the comparisons of *col-5* and *sdd1-2* either in monoculture (same genotype) or in a mixture (*col-5* and *sdd1-2*).

	Source of variation	df	SS	F value	P
Genotype monoculture	***Watering regime***	1	3158.8	6.971	<0.05
Intra-genotypic competition	***Genotype***	1	1691.7	3.733	0.06
	Water regime x genotype	1	263	0.58	0.45
	Residuals	44	19937.3		
Genotype mixture	***Watering regime***	1	657.1	1.214	0.277
Inter-genotypic competition	***Genotype***	1	6643.9	12.271	<0.01
	Water regime x genotype	1	103.3	0.191	0.665
	Residuals	42	22739.4		

Table 6. 2. Two-factor ANOVA table for belowground biomass at time of flowering for *col-5* and *sdd1-2* growing in intra- and inter-genotypic competition under high water (HW) and water-limited (LW) conditions. The term genotype refers to the comparisons of *col-5* and *sdd1-2* either in monoculture (same genotype) or in a mixture (*col-5* and *sdd1-2*).

	Source of variation	df	SS	F value	P
Genotype monoculture	***Watering regime***	1	0.201	0.184	0.67
Intra-genotypic competition	***Genotype***	1	2.154	1.9789	0.167
	Water regime x genotype	1	1.059	0.973	0.329
	Residuals	43	46.812		
Genotype mixture	***Watering regime***	1	9.425	6.283	<0.05
Inter-genotypic competition	***Genotype***	1	12.378	8.251	<0.01
	Water regime x genotype	1	19.949	13.297	<0.01
	Residuals	42	1.5		

Aboveground to belowground biomass ratios in relation to water stress are presented and analysed in Figure 6. 2 and Table 6. 3 respectively. Figure 6. 2 shows that water stress (HW *versus* LW) diminished the allocation to aboveground biomass for both genotypes growing alone, in intra- and inter-genotypic competition. Analysis of variance indicated a significant three-way interaction reflecting the differences in allocation to above and belowground biomass of the *col-5* genotype in competition with *sdd1-2.*

6. 3. 2. RNE

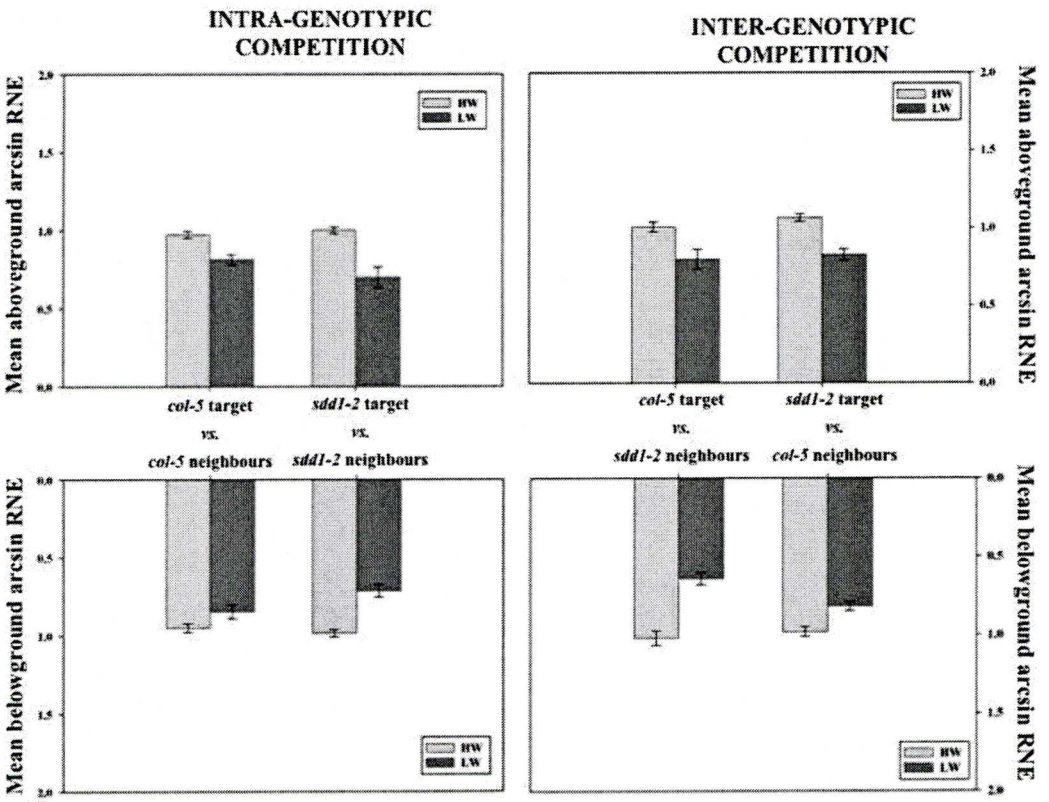

Figure 6. 3. The effects of intra- and inter-genotypic competition on the RNE at time of flowering under water regimes. Results are mean ± sem.

Values for RNE as calculated from equation 1 were always positive with the intensity of competition being greater under HW than LW (Figure 6. 5) as observed in Chapter 5. There were no significant differences due to the type of competition (P>0.005; Table 6. 4) considering aboveground biomass, although the mean RNE of *sdd1-2* in intra-genotypic competition was the lowest, the intensity of competition as assessed by RNE belowground was of a similar magnitude to that above-ground under HW. This intensity was reduced under LW (P<0.01; Table 6. 5) for both genotypes and *sdd1-2* exhibited a lower index under intra-genotypic competition than *col-5*. The inverse of this relationship was noticeably evident considering inter-genotypic competition. Least belowground competition was evident for *col-5* in the presence of *sdd1-2* neighbours,

relative to *sdd1-2* as target with *col-5* neighbours. This difference in mean responses underlay the significant third order interaction term (Table 6. 5).

Table 6. 4. Two-factor ANOVA of the RNE index for aboveground biomass. Factors were HW and LW, genotype *sdd1-2* and *col-5* and competition, plants grown under intra-genotypic competition and plants grown under inter-genotypic competition.

Source of variation	df	SS	F value	P
Water regime	1	1.314	100.362	<0.01
Genotype	1	0.015	1.182	0.28
Competition	1	0.046	3.491	0.065
Water x genotype	1	0.022	1.67	0.200
Water x competition	1	<0.01	0.049	0.825
Genotype x competition	1	0.048	3.701	0.058
Water x genotype x competition	1	0.032	2.445	0.122
Residuals	86	1.126		

Table 6. 5. Two-factor ANOVA of the RNE index for belowground biomass. Factors were HW and LW, genotype *sdd1-2* and *col-5* and competition, plants grown under intra-genotypic competition and plants grown under inter-genotypic competition.

Source of variation	df	SS	F value	P
Water regime	1	1.193	92.967	<0.01
Genotype	1	0.017	1.344	0.249
Competition	1	<0.01	0.078	0.782
Water x genotype	1	0.002	0.138	0.711
Water x competition	1	0.025	1.964	0.165
Genotype x competition	1	0.045	3.488	0.065
Water x genotype x competition	1	0.175	13.681	<0.01
Residuals	85	1.09		

6. 3. 3. Flowering time

Table 6. 6. Codes used in the analysis of flowering from the competition experiment

Code	TARGET	NEIGHBOUR	WATER REGIME
CCHW	*col-5*	*col-5*	HW
SSHW	*sdd1-2*	*sdd1-2*	HW
CSHW	*col-5*	*sdd1-2*	HW
SCHW	*sdd1-2*	*col-5*	HW
CCLW	*col-5*	*col-5*	LW
SSLW	*sdd1-2*	*sdd1-2*	LW
CSLW	*col-5*	*sdd1-2*	LW
SCLW	*sdd1-2*	*col-5*	LW

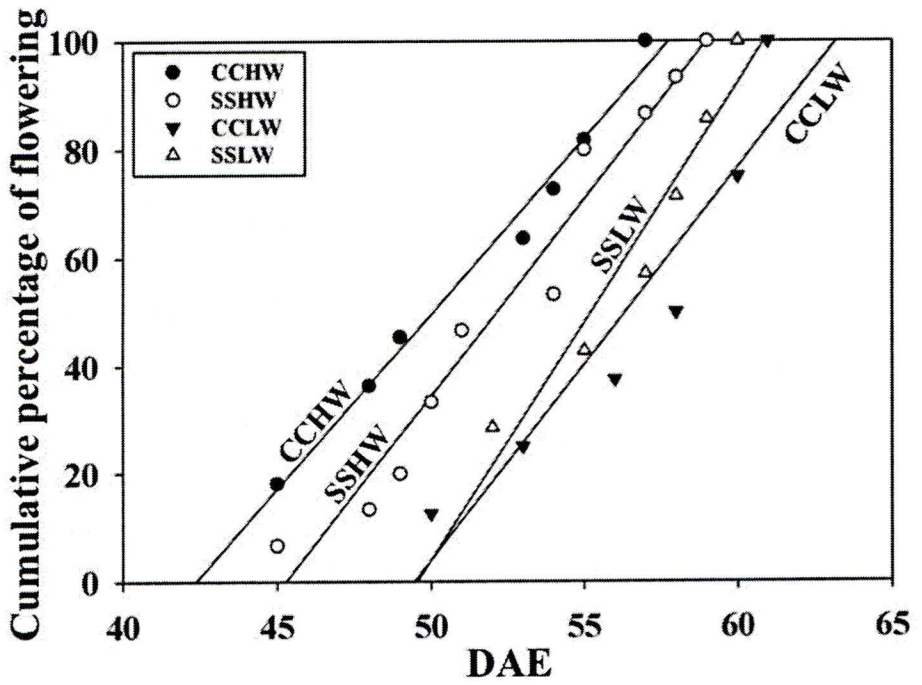

Figure 6. 4. Cumulative percentage of flowering against time (DAE) for *col-5* and *sdd1-2* grown in monoculture under HW and LW.

Table 6. 7. ANOVA of number of the cumulative percentage of flowering in relation to time in *col-5* and *sdd1-2* when grown in monoculture under HW and LW.

Source of variation	df	SS	F value	P
Watering regime (HW/LW)	1	9.7	0.161	0.693
Time (DAE)	1	22805	379.439	<0.01
Genotype	1	85	1.415	0.247
Water x time (DAE)	1	107.7	1.792	0.195
Water x genotype	1	990.8	16.485	<0.01
DAE x genotype	1	105.1	1.749	0.2
Water x DAE x genotype	1	11.8	0.197	0.662
Residuals	21	1262.2		

Table 6. 8. Linear regression (Equation 3, Chapter 4) of cumulative percentage of flowering and time (DAE) for *col-5* and *sdd1-2* in relation to watering regimes growing in monoculture.

Treatment	Relative flowering rate	Standard error	P value	r^2
CCHW	6.487	0.351	<0.01	0.98
SSHW	7.291	0.492	<0.01	0.968
CCLW	7.276	1.277	<0.01	0.89
SSLW	8.838	1.147	<0.01	0.937

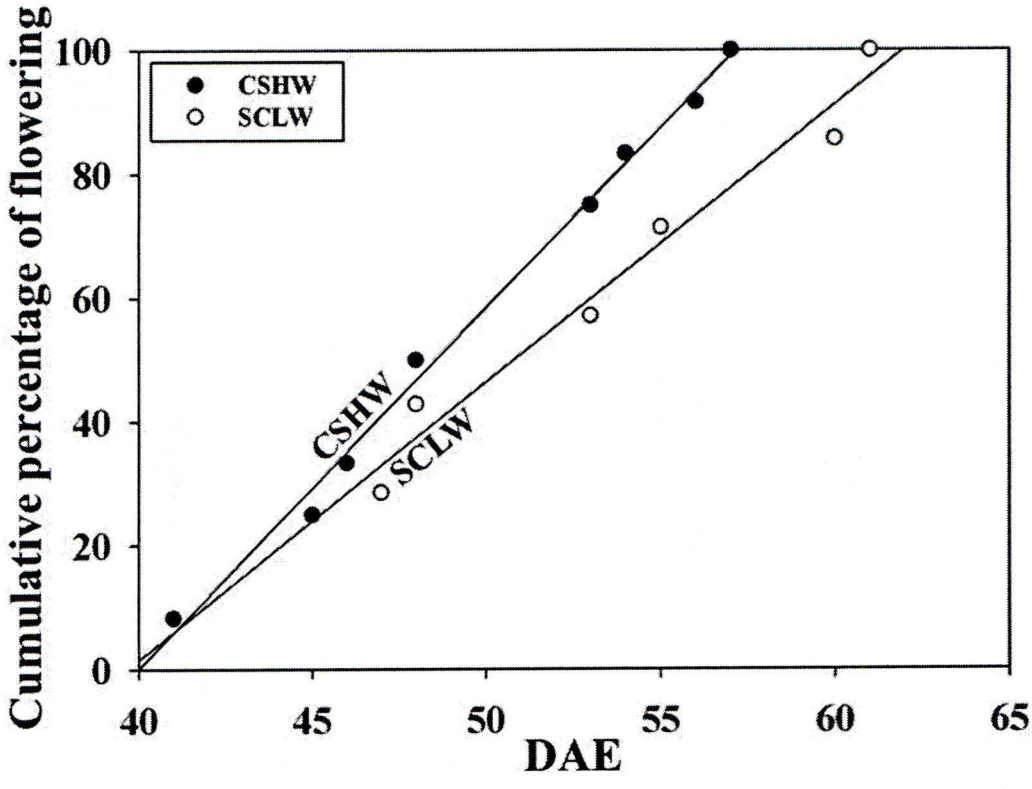

Figure 6. 5. Cumulative percentage of flowering against time (DAE) of *col-5* growing in the presence of *sdd1-2* under HW and *sdd1-2* growing in the presence of *col-5* under LW.

Table 6. 9. Linear regression of cumulative percentage of flowering against time (DAE) using a linear fit for *col-5* under HW and *sdd1-2* under LW growing in mixture with individuals of the opposite genotype.

Treatment	Relative flowering rate	Standard error	P value	r^2
CSHW	5.826	0.177	<0.01	0.98
SCLW	4.487	0.403	<0.01	0.937

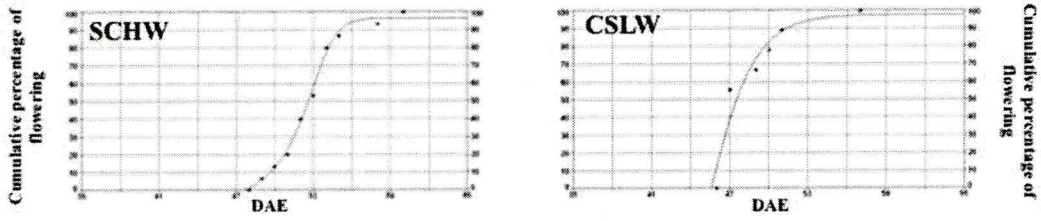

Figure 6. 6. Cumulative percentage of flowering against time (DAE) of *sdd1-2* growing in the presence of *col-5* under HW and *col-5* growing in the presence of *sdd1-2* under LW using a reverse asymmetric sigmoid function.

Table 6. 10. Cumulative percentage of flowering against time (DAE) of *sdd1-2* growing in the presence of *col-5* under HW and *col-5* growing in the presence of *sdd1-2* under LW. This table present the model fit using a reverse asymmetric sigmoid function (Chapter 4, equation 4).

Treatment	a	b	c	d	e	P value	r^2
***sdd1-2* HW**	-7.268	103.795	52.398	0.792	0.338	<0.01	0.995
***col-5* LW**	-64.331	161.947	46.198	1.976	<0.01	0.157	0.92

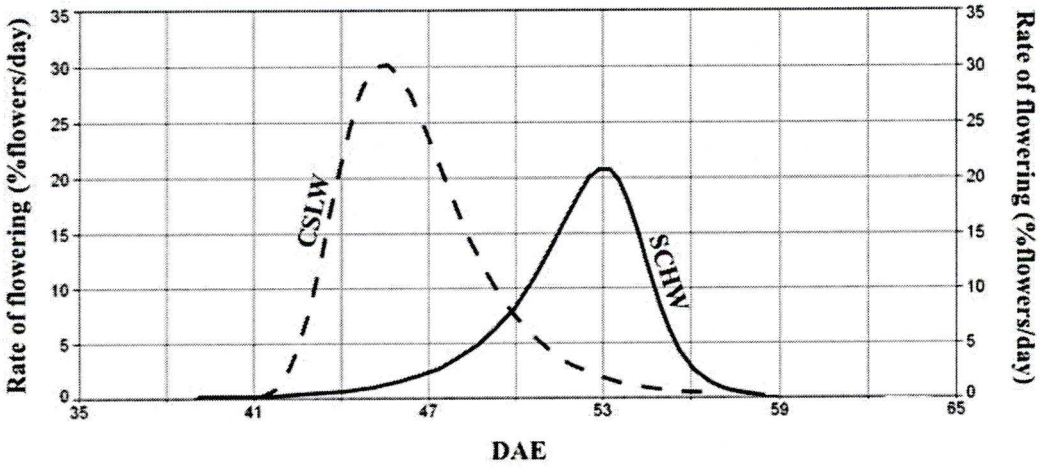

Figure 6. 7. Rate of flowering calculated as the first derivative of the cummulative percentage of flowering against time (DAE) of *sdd1-2* growing in the presence of *col-5* under HW and *col-5* growing in the presence of *sdd1-2* under LW.

Table 6. 6 presents the analysis of flowering rate in monocultures of each genotype in relation to watering regimes and indicates that rates of flowering were significantly different ($P<0.01$). *Sdd1-2* flowered at a faster rate than *col-5* under both watering regimes (Figure 6. 4, Table 6. 7), the fastest rate overall being measured for *sdd1-2* under LW.

In monogenotypic stands under HW (Figure 6. 4, Table 6. 7 and 6. 8), *col-5* showed 6.48 % flowers per day (Table 6. 7) in comparison to 7.29 *sdd1-2* (Table 6. 8). Flowering started at the same point in time (45 DAE), with the wild-type completing the 100% of cumulative flowering at 57 DAE (12 days) while *sdd1-2* reached that point after 59 DAE (14 days). The average time of flowering was 51 days for *col-5* and 53 for *sdd1-2*. Under intra-genotypic conditions in the absence of water-limitation, both genotypes behaved very similarly.

Monogenetic stands under water-limited conditions (Figure 6. 4, Table 6. 7 and 6. 8); *col-5* started flowering at 50 DAE until 51 DAE (11 days) while flowering in *sdd1-2* continued from 52 to 60 DAE (8 DAE). Average flowering time was 57 for the wild-type and 56 for the mutant. The effect of water-limitation on flowering time was to delay rather than accelerate their flowering. The gap of time during which flowering occurs under intra-genotypic competition is reduced by water limitation from 12 and 14 days for *col-5* and mutant respectively to 11 and 8 days under water-limited conditions.

Rates of flowering over time were observed to be linear for *col-5* versus *sdd1-2* under HW and *sdd1-2* versus *col-5* under LW (Figure 6. 5) but non-linear for the reverse genotypic combinations (Figure 6. 6).

Plants competing with those of the opposite genotype (Figures 6. 5, 6. 6 and 6. 7, Tables 6. 9 and 6. 10) under HW differed in their flowering periods. Wild-type started flowering earlier (41 DAE) than *sdd1-2* (49 DAE) and last flower was visible earlier (57 vs. 60 DAE) giving a wider flowering window (16 days) than *sdd1-2* (11 days). Average flowering was 50 DAE for *col-5* and 53 DAE for *sdd1-2*. The wild-type constant flowering rate of 5.82 % flowers/day was lower than that measured in monogenetic treatments. The flowering rate of *sdd1-2* under HW peaked at with 51 % of plants flowering per day.

Under WL competing intergenotypically (Figures 6. 5, 6. 6 and 6. 7; Table 6. 9 and 6. 10), showed a variable rate of flowering whilst *sdd1-2* presented a linear response with a low but constant flowering rate of 4.487 % of plants reaching the flowering stage per day. *Sdd1-2* started flowering at 47 DAE; showing flowering until 61 DAE (14 days) while last individual to flower in the wild-type did so at 57 DAE (12 days). On average, *col-5* plant flowered 51 DAE for the 53 DAE of the average date in which the stomatal density mutant would switch from vegetative to reproductive growth. *Col-5* flowered at a maximal rate around 45 DAE, the narrower temporal window being compensated by attaining higher flowering rates of 30%.

Thus, water-limitation under inter-genotypic competition did not clearly affect the average flowering time, As for the length of the flowering period, the trend changes depending on the genotype. For *col-5* in intra-genotypic competition, water-limitation reduced the flowering period by 6 days (16 under well-watered and 10 days water-limited conditions). However, for the stomatal density mutant, water limitation expanded the flowering period by two days (11 days under well-watered and 14 day under water-limited conditions).

6. 4. Discussion

The experiment reported in this Chapter was designed to explore in greater depth the competitive interactions described in Chapter 5, albeit at a constant overall density and with the constraints of a rectangular planting arrangment.

As plants growing alone (Figure 4. 2, Chapter 4), *col-5* was larger than *sdd1-2* at time of flowering and water limitation decreased aboveground and belowground biomass but no significant interaction between genotype and water treatment was found. Under intra-genotypic competition (Figure 6. 3) neither genotype did better than the other competing with plants of the same genetic background; additionally the water limitation reduced aboveground biomass but not nearly as drastically as it was with plants growing alone. This suggests that alterations in stomatal density did not have an effect on plant growth in an intra-genotypic stand in these experimental conditions and that the effect of the alteration of watering regime was reduced in a stand of plants. One possible explanation for this may be that as aboveground plant cover increases it reduces

evapotranspiration from the soil. Whilst the increased number of individuals also implies a larger demand for the limiting resource, water conservation due to reduced evapotranspiration may be a larger factor compared to resource loss by increase water uptake by a larger number of individuals. It also suggest that as plant density increases, the effect of water stress on competition leads way for competition for "biological space" (Cornforth 1968; Ross & Harper 1972; McConnaughay & Bazzaz 1991) interpreted as a plant that occupy a given area of land rather than as individuals assembled in populations (Harper 1976).

Considering inter-genotypic interactions, the wild-type grows slightly better aboveground while not growing differently belowground. But under water limited conditions, wild-type presented a considerably larger biomass production aboveground and specially belowground compared to the stomatal density mutant (Figure 6. 3). So the different WUE of *col-5* plants growing alone described in Chapter 4 did not play a role on intra-genotypic competition did became a competitive advantage when competing with *sdd1-2* plants with a reduced WUE.

Water limitation produced a decrease in aboveground to belowground ratios as observed in other studies (Sharp & Davies 1985; Larcher 1995; Taiz & Zeiger 2006). This held true for plants growing alone as well as in intra- and inter-genotypic competition. Under intra-genotypic competition in HW, *col-5* allocated more biomass than *sdd1-2*. This represents the optimal strategy for competition under these conditions because since there is no limitation on belowground resources, and competition will be mainly for light, assuming no other resource limitations is going to be mainly for light. However, under water-limited conditions when plants compete for belowground resources, *col-5* is the genotype that allocates more resources to belowground growth may be advanted. Shoot growth may be limited by water uptake in the same reciprocal manner that root growth is dependent upon photosynthetic rate and source-sink relationships. Thus, root exploration of the soil will be limited by the effects of water limitation on carbon capture in photosynthesis. Arguably the reduced WUE of *sdd1-2* compared to *col-5* described in Chapter 4 is responsible for this lesser plasticity in resource allocation in the mutant in response of water availability.

In ecological studies, the result of a competitive interaction is frequently measured as the ratio of relevant performance variables (i.e. biomass, reproductive fitness...), among individuals with neighbors and plants growing alone. Several relevant response variables can be expressed as an absolute value. The choice of these variables has a major impact on the assessment of comparisons of the magnitude of competition as we have seen from the assessment of aboveground and belowground biomass. But competition cannot be determined simply by biomass production unless competitive effects are linear (Goldberg & Scheider 2001). If absolute values for growth (such as biomass) under competition are chosen, this does not take into account the fact that the mutant also has a lower growth than its wild-type growing by itself. Thus, a mutant would not be considered necessarily a worse competitor because it shows a lesser absolute decrease in growth growing in a high plant density stand. However, taking growth as a ratio such as the one proposed with the use of RNE, the index of competition takes this factor into account.

Water limitation reduced RNE for both genotypes under intra- and inter-genotypic competition, and this may be due to the fact that as biomass decreases, direct competition for light decreases and is substituted by indirect competition for common limited resources, which are related to water availability. Under both intra- and inter-genotypic competition in HW no differences in RNE were found between genotypes

Results from the RNE analysis together with those obtained from the study of biomass described in Chapter 4, suggest that under well-watered conditions, the competitive ability of *sdd1-2* and growth were similar to those attained by *col-5* both when competing intra- or inter-genotypically. When water was reduced both genotypes growing in monogenetic stands present similar growth. Monogenetic *col-5* stands present a higher competitive interaction than *sdd1-2*, genotype with a lesser WUE (Chapter 4). When this is translated to an inter-genotypic competitive event where both genotypes are competing for water; this higher competitive ability shown by *col-5* translates into a higher growth.

For plants growing in the absence of competition, reduction of water delayed the point of time at which plants reached flowering, the duration of which took place over a narrower period of time. Under intra-genotypic conditions in the absence of water-

limitation, both genotypes behaved very similarly. The effect of water-limitation on flowering time was similar to that of plants growing alone and to delayed their flowering. Conversely, inter-genotypic competition delayed flowering for both genotypes under both watering regimes. It also resulted with a larger window of flowering than plants growing alone. Whilst water-limitation under inter-genotypic competition did not clearly affect the average flowering time, water limitation aligned the peaks of maximal flowering rates in both genotypes more closely.

Two hypotheses have been proposed to explain the relationship between size and flowering time in *A. thaliana*. Lacey (1986) and Schmitt *et al.* (1986) argued that plants growing alone will flower later than plants growing in dense stands purely because of size. Alternatively plants grown at high densities may flower later because they will be smaller (Clay & Shaw 1981) and therefore will take longer to attain the minimum or "critical" size necessary to be able to flower (Werner 1975) even though they may end up flowering at a smaller size that plants growing alone. Since the intensity of competition can change such that different resources assume different levels of importance in different conditions (Aerts 1999) which may occur across a density range. Results from this work agree with Clay and Shaw (1981) since plants growing alone reached the reproductive stage earlier than plants growing in intra- and inter-genotypic competition. *A. thaliana* plants growing in a dense stand take longer to reach their minimum size to flower.

However this was also true in the case of water limitation under intra-genotypic competition. Water-limitation reduced RGR as described in Chapter 4 and further delayed the start of the reproductive stage. Inter-genotypic competition altered this relationship. Both genotypes, with different growth patterns competing for water as a limited resource, triggered flowering earlier in both instances.

The acquisition of a competitive ability may come with a trade-off in which there is a cost evident under competition in different environmental conditions (Blossey & Notzold 1995) There has been a long debate in ecology about the intensity and importance of competition along resource gradients and whether plant attributes assume different levels of importance in different habitats (Aerts 1999). Grime (2001) predicted that competition intensity should remain constant along productivity gradients whilst on

the other hand Tilman (1988) considered that it should change along different productivity gradient. This study agrees with Tilman's prediction in that the genotype with a lower water use efficiency, attained similar competitive ability under well-watered conditions while being a less efficient competitor as water became a limiting resource

Chapter 7. General discussion

Interest in studies on *Arabidopsis thaliana,* both as an invasive annual species and given that it is a model species, have been increasing in recent years (Hoffmann *et al.* 2003; Kolodynska & Pigliucci 2003; McKay, Richards & Mitchell-Olds 2003; Donohue *et al.* 2005; Bakker *et al.* 2006; Blodner *et al.* 2007; Ghalambor *et al.* 2007; Moyers & Kane 2010). The research in this thesis has attempted a comprehensive analysis of genotypes of *A. thaliana* with the objective of assessing the consequences of a change in a single morphological trait, stomatal density, on leaf phytochemistry, photosynthesis and water use, growth and flowering and the implications for competitive interactions. Whilst studies utilised several *Arabidopis* mutants, the emphasis was placed on comparative assessment of *col-5* and *sdd1-2* to provide critical insights into underlying phenotypic trait differences.

The monocarpic life history of *A.thaliana* lends itself to detailed analysis of the physiological and ecological characteristics of the species because of the distinct growth stages and the relatively abrupt transition from vegetative to reproductive growth. The size of the vegetative plant relates to the reproductive potential of the individual as the number of reproductive spikes (bolts) is related to biomass, as are the numbers of flowers, but in an indeterminate manner as there is no terminal meristem on the flowering axis. Work here therefore focussed on the relative performance of *Arabidopsis* genotypes at tractable points in the life cycle. The use of two water regimes, that were empirically chosen, was discriminating in that phenotypic differences between genotypes were experimentally detectable. In particular the different water regimes (frequency of watering during the majority of the vegetative stage) had a noticeable effect on reproductive performance as evidenced in particular by the preliminary observations on the effects of soil volumes (Chapter 2), allometric relationships (Chapter III) and competitive interactions (Chapter 5 and 6). The results, and conclusions drawn from them, however are specific to the soil media that was used and the choice of a sand compost mix, as opposed to a particular compost. The addition of sand improved growth by increasing the aeration of the soil and additionally facilitated experimental work when for root extraction. Proof of the improvement made with the use of flats in the

main trials and with the addition of sand to the compost was the fact that as described in Chapter 3 , the vegetative biomass for *col-0* never exceeded 0.2 g, whilst in the study of genotypic responses to density in Chapter 4, isolated plants were predicted to achieve 0.35 g in biomass. The depth of soil employed in the flats (55mm deep) will have also influenced root foraging strategy.

One of the novel parts of this study came with the opportunity to make single-leaf measurements of photosynthesis using a Licor-6400 with an incorporated fluorometer. The small leaf areas and petiole size of *A. thaliana* have previously prevented detailed individual leaf measurements but this was successfully achieved. This enabled measurement of key photosynthetic parameters whilst stomata units were wide open or in other words, in the absence of stomatal regulation of gaseous exchange. This has not been reported in the literature to date.

Measurements on the phytochemistry of the *sdd1-2* mutant and its wild-type *col-5* were undertaken to investigate the possibility of differences in leaf traits in terms of boundary layer resistance, stomatal resistance and mesophyll resistance (Chapter 2). No statistically significant differences were detected in the chlorophyll fluorescence parameters that characterize the photobiology of plants. From F_v/ F_m^0 ratios were close to 0.8, suggesting that photo-inhibition not occurring at steady-state conditions in these studies. This indicates that the growth conditions were optimal as suggested in our preliminary growth studies and thus that the conditions used in subsequent Chapters as the optimal/well-watered conditions were indeed optimal Characterisation of A/Ci curves and points of light saturation was also made to assess whether there was stomatal limitation of CO_2 uptake (Farquhar & Von Caemmerer 1982; Sharkey *et al.* 2007). The conclusion from these studies was that differences in gas exchange between mutant and wild-type were likely to be due solely to differences in stomatal conductance and not due to a different mesophyll conductance or alteration in the photobiochemistry of the plant. This suggests that there were no pleiotropic genetic effects in the *sdd1-2* allele mutation of the *SDD1* mutation and supports the findings of Schlüter *et al.*, (2003) who concluded that the mutant *sdd1-1* and wild-type, had similar photosynthetic responses. The *sdd1-2* phenotype can therefore be considered to have a phenotype with an elevated stomatal density up to four fold compared to the wild-type with similarity on internal

leaf architecture of the *sdd1-2* leaves do not differ from wild-type (Berger & Altmann 2000).

Stomatal density clearly plays an important role in the water use efficiency of higher plants (Woodward 1987; Woodward & Bazzaz 1988; Mansfield, Hetherington & Atkinson 1990) and in determining the rate carbon capture since stomatal density determines maximum stomatal conductance that a leaf presents per unit area (Drake, Gonzalez-Meler & Long 1997b). Stomatal conductance is. Stomatal density affects the response of plants to drought conditions (El-Sharkawy, Cock & Hernandez 1985) and arguably is optimized over the long-term (through life history and evolutionarily) through developmental changes in stomatal density.Periods of low atmospheric CO_2 concentrations in the past have been associated with an increase in stomatal densities in leaves (Woodward 1987; Beerling & Woodward 1997; Beerling, McElwain & Osborne 1998; Hetherington & Woodward 2003). Leaf water use efficiency increased through these periods in concert with the reduced stomatal density (Woodward 1993) so variations in atmospheric CO_2 may lead to evolution in stomatal density in plants - of relevance to future global environments given projected increases in atmospheric CO_2 levels. Results in Chapter 4 show that the wild type phenotype *col-5* reduced stomatal density in comparison to *sdd1-2* was able to capture the same amount of CO_2 whilst losing less water, under well-watered conditions. Subsequent experiments investigated the consequences of a lowering of water (and indirectly soil nutrient) availability on performance of these genotypes as isolated plants and in competition.

Plant species can adjust their phenology depending on the environmental conditions, particularly water availability. It was therefore interesting to study the effect of the likely trade-off between carbon gain and water loss at differential stomatal density on plant growth under optimal conditions compared to water-limited conditions. Not-surprisingly, growth rates on aboveground biomass were higher under well-watered conditions (HW) than under water-limited conditions (LW), as imposed here. Since stomatal conductance was higher and water use efficiency was lower in the *sdd1-2* genotype, the null hypothesis was that *sdd1-2* would show a reduced growth rate compared to *col-5* under LW. Under water-limited conditions *sdd1-2* grew more slowly

than *col-5*, a difference but again this was not significantly different, such that both genotypes reduced their growth at the same rate.

Relative growth rates of roots of *A. thaliana* are stimulated by water limitation (Van Der Weele *et al.* 2000) and it is well known, that in plants growing in a drying soil, with a low water potential, root growth is less inhibited or even enhanced in relation to aboveground growth (Sharp & Davies 1979; Westgate & Boyer 1985). At the molecular level, it has been proposed that abscisic acid plays an important role in the response of roots to low water potentials in the soil by limiting ethylene production in the roots (Sharp & LeNoble 2002; Sharp *et al.* 2004) and thus explaining the independent growth response of aboveground and belowground portions of plants. Results from this work however suggested that *col-5* allocated more resources to belowground growth than *sdd1-2*. and given that *sdd1-2* has a higher stomatal density it might have been expected that it would exhibit a higher belowground RGR than *col-5*.

The literature, indicates that studies on the relationship between stomatal densities and leaf hydraulics has not received much attention in the past. Clearly there is potential to utilise *A. thaliana* as a model, and take advantage of the diversity of mutants and ecotypes available with respect to stomatal density and other leaf traits. This may have a particular bearing on breeding for improved water use in crops. Percy *et al.* (1996) suggested that higher stomatal conductance in Pima cotton may produce increased yield, and thus proposed that there would be a benefit from selecting crops for this trait. For the same species, Lu and Zeiger (1994) found that decreased stomatal density could also increase yield in water-limited conditions.

Grime (1977) suggested that species with a higher relative growth rate would be better competitors because rapid growth enabled plants to acquire more resources. On the other hand, Tilman (1988) argued that species able to uptake reduced levels of a limiting resource would possess the competitive advantage. Combining both ideas, Grace (1990) considered that both theories were not incompatible and that when plant growth occurred over a short-time span as in the case with *A. thaliana* in disturbed environments RGR will contribute in a major proportion to competitive success. However, in dense plant communities, the potential of a species to exploit the resources will determine its competitive success. The results present in Chapter 5 and 6 support

this view. In the absence of density dependent mortality, the growth and yield of *A. thaliana* was strongly density dependent, but individual plant yield was also very variable. Comparing the findings of the two methodological approaches to intra-and inter-genotypic competition, it was evident that *sdd1-2* was more sensitive to inter-genotypic competition than *col-5* and this in turn was dependent on watering regime. Experimental findings were however tantalising in that responses at high density did not reduce yield as much as might be predicted from conventional yield models (Chapter IV). Investigations at constant lower overall density (Chapter 6) suggested that the intensity of intra- and inter-genotypic competition was similar in both genotypes, considering aboveground biomass at point of flowering, inter-genotypic effects were detectable for root biomass, with the wild-type being less sensitive than the mutant.

Phenotypic plasticity, the capacity of a given genotype to render different phenotypic values for a given trait under different environmental conditions is well known in *A. thaliana* plasticity (Jones, 1971; (Westerman & Lawrence 1970; Zhang & Lechowicz 1994; Zhang & Forde 1998; Stinchcombe, Dorn & Schmitt 2004b; Weining *et al.* 2006) In the last years there is an increasing interest in the study of plant plasticity in order to predict species responses to global change (Potvin & Tousignant 1996; Valladares, Sanchez-Gomez & Zavala 2006) As pointed out by Callaway et al. (2003), the understanding of the evolutionary ecology of phenotypic plasticity is rapidly advancing (particularly in *A.thaliana*), but little is known about the contribution of phenotypic plasticity for ecological interactions such as competition or facilitation. A final observation from the studies presented in this thesis is that despite the use of defined genotypes and controlled environmental conditions, the demonstrable intrinsic phenotypic variability of this species constitutes both a challenge for future studies and an opportunity to investigate causal mechanisms that may be strongly influenced by rooting responses to the soil environment.

Literature cited

Aarssen, L.W. & Clauss, M.J. (1992) Genotypic variation in fecundity allocation in *Arabidopsis thaliana*. *Journal of Ecology,* **80,** 109-114.

Abdolzadeh, A., Wang, X., Veneklaas, E.J. & Lambers, H. (2010) Effects of phosphorus supply on growth, phosphate concentration and cluster-root formation in three Lupinus species. *Annals of Botany,* **105,** 365-374.

Aerts, R. (1999) Interspecific competition in natural plant communities: mechanisms, trade-offs and plant-soil feedbacks. *Journal of Experimental Botany,* **50,** 29-37.

Al-Shehbaz, I.A. & O'Kane, S.L. (2002) Taxonomy and Phylogeny of *Arabidopsis* (Brassicaceae). *The Arabidopsis Book.*

Alpert, P. (2000) The discovery, scope, and puzzle of desiccation tolerance in plants. *Plant ecology,* **151,** 5-17.

Alwerdt, J.L., Gibson, D.J., Ebbs, S.D. & Wood, A.J. (2006) Intraspecific interactions in *Arabidopsis thaliana* and the stomatal mutants *tmm1-1* and *sdd1-2*. *Biologia Plantarum,* **50,** 205-209.

Andalo, C., Goldringer, I. & Godelle, B. (2001) Inter- and intragenotypic competition under elevated carbon dioxide in *Arabidopsis thaliana*. *Ecology,* **82,** 157-164.

Araus, J., Alegre, L., Tapia, L., Calafell, R. & Serret, M. (1986) Relationships between photosynthetic capacity and leaf structure in several shade plants. *American Journal of Botany*, 1760-1770.

Bakker, E.G., Stahl, E.A., Toomajian, C., Nordborg, M., Kreitman, M. & Bergelson, J. (2006) Distribution of genetic variation within and among local populations of *Arabidopsis thaliana* over its species range. *Molecular Ecology,* **15,** 1405-1418.

Ballaré, C. & Scopel, A. (1997) Phytochrome signalling in plant canopies: testing its population-level implications with photoreceptor mutants of *Arabidopsis*. *Functional Ecology,* **11,** 441-450.

Baskin, J. & Baskin, C. (1983) Seasonal changes in the germination responses of buried seeds of *Arabidopsis thaliana* and ecological interpretation. *Botanical Gazette*, 540-543.

Bates, T.R. & Lynch, J.P. (2001) Root hairs confer a competitive advantage under low phosphorus availability. *Plant and Soil,* **236,** 243-250.

Beerling, D., McElwain, J. & Osborne, C. (1998) Short communication. Stomatal responses of the 'living fossil' *Ginkgo biloba L.* to changes in atmospheric CO_2 concentrations. pp. 1603-1607.

Beerling, D.J. & Woodward, F.I. (1997) Changes in land plant function over the phanerozoic: reconstructions based on the fossil record. *Bot. J. Linn. Soc.,* **124,** 137-153.

Bentsink, L. & Koornneef, M. (2002) Seed dormancy and germination. *The Arabidopsis Book* (eds L. Bentsink & M. Koornneef), pp. 1-18. ASCP.

Berardini, T., Bollman, K., Sun, H. & Scott Poethig, R. (2001) Regulation of vegetative phase change in *Arabidopsis thaliana* by cyclophilin 40. *Science,* **291,** 2405.

Berger, D. & Altmann, T. (2000) A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. *Genes and Development,* **14,** 1119-1131.

Bergmann, D.C. (2004) Integrating signals in stomatal development. *Current Opinion in Plant Biology,* **7,** 26-32.

Bergmann, D.C., Lukowitz, W. & Somerville, C.R. (2004) Stomatal Development and Pattern Controlled by a MAPKK Kinase. *Science,* **304,** 1494-1497.

Bernacchi, C., Portis, A., Nakano, H., von Caemmerer, S. & Long, S. (2002) Temperature response of mesophyll conductance. implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo. *Plant physiology,* **130,** 1992-1998.

Berry, T.A. & Bewley, J.D. (1992) A role for the surrounding fruit tissues in preventing germination of tomato (*Lycopersicon esculentum*) seeds. A consideration of the osmotic environment and abscisic acid. *Plant physiology,* **100**.

Bewley, J.D. (1997) Seed germination and dormancy. *The Plant Cell,* **9,** 1055-1066.

Bilger, W. & Björkman, O. (1990) Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. *Photosynthesis Research,* **25,** 173-185.

Björkman, O. & Demmig, B. (1987) Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta,* **170,** 489-504.

Blackman, V.H. (1919) The compound interest law and plant growth. *Annals of Botany,* **33,** 353-360.

Bleasdale, J. & Nelder, J. (1960) Plant population and crop yield.

Blodner, C., Goebel, C., Feussner, I., Gatz, C. & Polle, A. (2007) Warm and cold parental reproductive environments affect seed properties, fitness, and cold responsiveness in *Arabidopsis thaliana* progenies. *Plan, Cell and Environment,* **30,** 165-175.

Bloom, A.J., Chapin, F.S. & Mooney, H. (1985) Reource limitation in plants-An economic analogy. *Ann. Rev. Ecol. Sys,* **16,** 363-392.

Blossey, B. & Notzold, R. (1995) Evolution of increased competitive ability in invasive nonindigenouse plants: a hypothesis. *Journal of Ecology,* **83,** 997-889.

Bolhar-Nordenkampf, H., Long, S., Baker, N., Oquist, G., Schreiber, U. & Lechner, E. (1989) Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Functional Ecology,* 497-514.

Boss, P.K., Bastow, R.M., Mylne, J.S. & Dean, C. (2004) Multiple Pathways in the Decision to Flower: Enabling, Promoting, and Resetting. *The Plant Cell,* **16,** S18-31.

Boyes, D., Zayed, A., Ascenzi, R., McCaskill, A., Hoffman, N., Davis, K. & Gorlach, J. (2001a) Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. *The Plant Cell Online,* **13,** 1499.

Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R. & Gorlach, J. (2001b) Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. *The Plant Cell,* **13,** 1499-1510.

Brestic, M., Cornic, G., Freyer, M. & Baker, N. (1995) Does photorespiration protect the photosynthetic apparatus in French bean leaves from photoinhibition during drought stress? *Planta,* **196,** 450-457.

Brodribb, T., Feild, T. & Jordan, G. (2007) Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant physiology,* **144,** 1890.

Brodribb, T., McAdam, S., Jordan, G. & Feild, T. (2009) Evolution of stomatal responsiveness to CO_2 and optimization of water-use efficiency among land plants. *New Phytologist,* **183,** 839-847.

Brown, R. (2002) Table Curve 2D v5. 01. *Automated curve fitting and equation discovery.*

Bullock, J., Mortimer, A. & Begon, M. (1994) The Effect of Clipping on Interclonal Competition in the Grass Holcus Lanatus--A Response Surface Analysis. *Journal of Ecology,* **82,** 259-270.

Bunce, J. (2006) How do leaf hydraulics limit stomatal conductance at high water vapour pressure deficits.

Bussis, D., von Groll, U., Fisahn, J. & Altmann, T. (2006) Stomatal aperture can compensate altered stomatal density in *Arabidopsis thaliana* at growth light conditions. *Functional plant biology,* **33,** 1037.

Cahill, J.F., Kembel, S.W. & Gustafson, D.J. (2005) Differential genetic influences on competitive effect and response in *Arabidopsis thaliana*. *Journal of Ecology,* **93,** 958-967.

Callahan, H. & Pigliucci, M. (2002) Shade-induced plasticity and its ecological significance in wild populations of *Arabidopsis thaliana*. *Ecology,* **83,** 1965-1980.

Canaani, O. & Malkin, S. (1984) Physiological adaptation to a newly observed low light intensity state in intact leaves, resulting in extreme imbalance in excitation energy distribution between the two photosystems. *Biochimica et Biophysica Acta (BBA)-Bioenergetics,* **766,** 525-532.

Candela, H., Martínez-Laborda, A. & Luis Micol, J. (1999) Venation pattern formation in *Arabidopsis thaliana* vegetative leaves. *Developmental Biology,* **205,** 205-216.

Casper, B.B. & Jackson, R.B. (1997) Plant competition underground. *Annual Review of Ecology and systematics,* **28,** 545-570.

Caton, B., Cope, A. & Mortimer, M. (2003) Growth traits of diverse rice cultivars under severe competition: implications for screening for competitiveness. *Field Crops Research,* **83,** 157-172.

Causton, D. & Venus, J. (1981) *The biometry of plant growth.* London.

Chapin, F.S., Bloom, A.J., Field, C.B. & Waring, R.H. (1987) Plant responses to multiple environmental factors. *Bioscience,* **37,** 49-57.

Charlesworth, D. & Vekemans, X. (2005) How and when did *Arabidopsis thaliana* become highly self-fertilising. *Bioessays,* **27,** 472-476.

Clauss, M.J. & Aarssen, L.W. (1994) Phenotypic plasticity of size-fecundity relationships in *Arabidopsis thaliana*. *Journal of Ecology,* **82,** 447-455.

Clay, K. & Shaw, R. (1981) An experimental demonstration of density-dependent reproduction in a natural population of *Diamorpha smallii*, a rare annual. *Oecologia,* **51,** 1-6.

Connell, J.I. (1990) Apparent versus "real" Competition in plants. *Perspectives on plant competition* (eds J.B. Grace & D. Tilman), pp. 9-27. Academic Press, Inc., San Diego, California.

Cook, R.E. (1980) Germination and Size-Dependent Mortality in Viola blanda. *Oecologia,* **47,** 115-117.

Corbesier, L., Vicent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C. & coupland, G. (2007) FT Protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science,* **316,** 1030-1033.

Cornforth, I. (1968) Relationships between soil volume used by roots and nutrient accessibility. *European Journal of Soil Science,* **19,** 291-301.

Cornic, G. & Briantais, J. (1991) Partitioning of photosynthetic electron flow between CO_2 and O_2 reduction in a C3 leaf (Phaseolus vulgaris L.) at different CO_2 concentrations and during drought stress. *Planta,* **183,** 178-184.

Coupland, G. (1995) Genetic and environmental control of flowering time in Arabidopsis. *Trends in Genetics,* **11,** 393-397.

Damgaard, C. (2004) Inference from plant competition experiments: the effect of spatial covariance. *Oikos,* **107,** 225-230.

Damgaard, C. (2008) Plant competition experiments: testing hypotheses and estimating the probability of coexistence.

Damgaard, C. & Borksted, B. (2004) Transgenic insect resistant *Arabidopsis* may show chaotic population dynamic. *Ecological Complexity,* **1,** 261-265.

Darwin, C. (1858) *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life*. John Murray, London.

Demmig-Adams, B. & Adams III, W. (1992) Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Biology,* **43,** 599-626.

Dodd, A., Parkinson, K. & Webb, A. (2004) Independent circadian regulation of assimilation and stomatal conductance in the ztl-1 mutant of *Arabidopsis*. *New Phytologist,* **162,** 63-70.

Donahue, R., Poulson, M. & Edwards, G. (1997) A method for measuring whole plant photosynthesis in *Arabidopsis thaliana*. *Photosynthesis Research,* **52,** 263-269.

Donohue, K. (2002) Germination timing influences natural selection on life-history characters in *Arabidopsis thaliana*. *Ecology,* **83,** 1006-1016.

Donohue, K., Dorn, L., Griffith, C., Kim, E., Aguilera, A., Polisetty, C.R. & Schmitt, J. (2005) The evolutionary ecology of seed germination of *Arabidopsis thaliana*: variable natural selection on germination timing. *Evolution,* **59,** 758-770.

Drake, B., Gonzalez-Meler, M. & Long, S. (1997a) More efficient plants: a consequence of rising atmospheric CO_2? *Annual Review of Plant Biology,* **48,** 609-639.

Drake, B.G., Gonzalez-Meler, M.A. & Long, S.P. (1997b) More efficient plants: A consequence of rising atmospheric CO_2? *Annual Review of Plant Physiology and Plant Molecular Biology,* **48,** 609-639.

Dyer, A., R. & Rice, K.J. (1999) Effects of competition on resource availability and growth of a california bunchgrass. *Ecology,* **80,** 2697-2710.

Edwards, G. & Baker, N. (1993) Can CO_2 assimilation in maize leaves be predicted accurately from chlorophyll fluorescence analysis? *Photosynthesis Research,* **37,** 89-102.

Ehleringer, J. (1980) Leaf morphology and reflectance in relation to water and temperature stress. *Adaptation of plants to water and high temperature stress. New York: Wiley-Interscience,* 123-128.

Ehleringer, J. (1984) Ecology and ecophysiology of leaf pubescence in North American desert plants. *Biology and Chemistry of Plant Trichomes,* 113-132.

El-Sharkawy, M., Cock, J. & Hernandez, A. (1985) Stomatal response to air humidity and its relation to stomatal density in a wide range of warm climate species. *Photosynthesis Research,* **7,** 137-149.

Ellstrand, N. & Elam, D. (1993) Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics,* **24,** 217-242.

Esau, K. (1965) *Plant anatomy,* 2nd edn. John Wiley & Sons., New York.

Farquhar, G., Caemmerer, S. & Berry, J. (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C3 species. *Planta,* **149,** 78-90.

Farquhar, G. & Von Caemmerer, S. (1982) Modelling of photosynthetic response to environmental conditions. *Encyclopedia of plant physiology,* **12,** 549-587.

Farquhar, G.D. & Sharkey, T.D. (1982) Stomatal conductance and photosynthesis. *Ann. Rev. Plant Physiol.,* **33,** 317-345.

Ferrero-Serrano, Hild, A. & Mealor, B. (2010) Can invasive species enhance competitive ability and restoration potential in native grass populations? *Restoration Ecology,* **In Press.**

Ferrero-Serrano, Á., Collier, T., Hild, A., Mealor, B. & Smith, T. (2008) Combined impacts of native grass competition and introduced weevil herbivory on Canada Thistle (*Cirsium arvense*). *Rangeland Ecology & Management,* **61,** 529-534.

Ferrero-Serrano, A., Collier, T.R., Mealor, B.A., Hild, A.L. & Smith, T. (2006) Combination of stem-boring weevil and native grasses reduces root biomass of Canada Thistle in greenhouse experiment (Wyoming). *Ecological Restoration,* **24,** 190-221.

Fitter, A. & Fitter, R. (2002) Rapid changes in flowering time in British plants. *Science,* **296,** 1689.

Flexas, J., Escalona, J., Evain, S., Gulìas, J., Moya, I., Osmond, C. & Medrano, H. (2002) Steady-state chlorophyll fluorescence (F_s) measurements as a tool to follow variations of net CO_2 assimilation and stomatal conductance during water-stress in C3 plants. *Physiologia Plantarum,* **114,** 231-240.

Flexas, J., Escalona, J. & Medrano, H. (1999) Water stress induces different levels of photosynthesis and electron transport rate regulation in grapevines. *Plant Cell and Environment,* **22,** 39-48.

Flexas, J., Ortuno, M., Ribas-Carbo, M., Diaz-Espejo, A., Florez-Sarasa, I. & Medrano, H. (2007a) Mesophyll conductance to CO_2 in *Arabidopsis thaliana*. *New Phytologist,* **175,** 501-511.

Flexas, J., Ortuno, M.F., Ribas-Carbo, M., Diaz-Espejo, A., Florez-Sarasa, I.D. & Medrano, H. (2007b) Mesophyll conductance to CO_2 in *Arabidopsis thaliana*. pp. 501-511.

Fowler, N. (1986) The role of competition in plant communities in arid and semi-arid regions. *Annual Review of Ecology and Systematics,* **17,** 89-105.

Franks, P., Drake, P. & Froend, R. (2007) Anisohydric but isohydrodynamic: seasonally constant plant water potential gradient explained by a stomatal control mechanism incorporating variable plant hydraulic conductance. *Plant Cell and Environment,* **30,** 19.

Fridley, J., Grime, J. & Bilton, M. (2007) Genetic identity of interspecific neighbours mediates plant responses to competition and environmental variation in a species-rich grassland. *Journal of Ecology,* **95,** 908-915.

Gallaway, L.F. (2002) The effect of maternal phenology on offspring characters in the herbaceous plant *Campanula americana*. *Journal of Ecology,* **90,** 851-858.
Galloway, L.F. (2001) Parental environmental effects on life history in the herbaceous plant *Campanula americana*. *Ecology,* **82,** 2781-2789.
Gamage, H. & Jesson, L. (2007) Leaf heteroblasty is not an adaptation to shade: seedling anatomical and physiological responses to light. *New Zealand Journal of Ecology,* **31,** 245-254.
Geisler, M., Nadeau, J. & Sack, F.D. (2000) Oriented asymmetric divisions that generate the stomatal spacing pattern in *Arabidopsis* are disrupted by the too many mouths mutation. *The Plant Cell,* **12,** 2075-2086.
Geisler, M., Yang, M. & Sack, F.D. (1998) Divergent regulation of stomatal initiation and patterning in organ and suborgan regions of the *Arabidopsis* mutants too many mouths and four lips. *Planta,* **205,** 522-530.
Genty, B., Briantais, J. & Baker, N. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim Biophys Acta,* **990,** 87-92.
Ghalambor, C.K., McKay, J.K., Carroll, S.P. & Reznick, D.N. (2007) Adaptative versus non-adaptative phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology,* **21,** 394-407.
Goebel, K. (1900) *Organography of Plants: General organography*. Clarendon press.
Goldber, R.B. (1996) Competitive Ability: Definitions, Contingency and Correlated Traits. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **351,** 1377-1385.
Goldber, R.B., Paiva, G.d. & Yadegari, R. (1994) Plant embryogenesis: zygote to seed. *Science,* **28.**
Goldberg, D. & Barton, A. (1992) Patterns and consequences of interspecific competition in natural communities: a review of field experiments with plants. *Am Nat,* **139,** 771.
Goldberg, D., Turkington, R., Olsvig-Whittaker, L. & Dyer, A. (2008) Density dependence in an annual plant community: variation among life history stages.
Goldberg, D.E. & Fleetwood, L. (1987) Competitive effect and response in four annual plants. *Journal of Ecology,* **75,** 1131-1143.
Goldberg, D.E. & Novoplansky, A. (1997) On the Relative Importance of Competition in Unproductive Environments. *Journal of Ecology,* **84,** 409-418.
Goldberg, D.E. & Scheider, S.M. (2001) ANOVA and ANCOVA: field competition experiments. *Design and Analysis of Ecological Experiments* (eds S.M. Scheiner & J. Gurevitch). Oxford University Press.
Goldberg, D.E. & Werner, P.A. (1983) Equivalence of competitors in plant communities: a null hypothesis and field experimental approach. *American Journal of Botany,* **70,** 1098-1104.
Goldberg, R.B. & Landa, K. (1991) Competitive effect and response hierachies and correlated traits in the early stages of competition. *Journal of Ecology,* **79,** 1013-1030.
Gould, K. (1993) Leaf heteroblasty in *Pseudopanax crassifolius*: functional significance of leaf morphology and anatomy. *Annals of Botany,* **71,** 61.
Grace, J.B. (1995) On the Measurement of Plant Competition Intensity. *Oikos,* **76,** 305-308.

Grant, D., Peters, D., Beck, G. & Fraleigh, H. (2003) Influence of an exotic species, Acroptilon repens (L.) DC. on seedling emergence and growth of native grasses. *Plant ecology,* **166,** 157-166.

Grime, J.P. (1977) Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. *The American Naturalist,* **111,** 1169-1194.

Grime, J.P. (2001) *Plant Strategies, Vegetation Processes and Ecosystem Properties*. John Wiley, Chichester.

Grime, J.P. & Hunt, R. (1975) Relative growth rate: its range and adaptive significance in a local flora. *Journal of Ecology,* **63,** 393-422.

Gross, K.L. & Smith, A.D. (1991) Seed mass and emergence time effects on performance of *Panicum dichotomitlorum* Michx. across environments. *Oecologia,* **87,** 270-278.

Gutterman, Y. & Boeken, B. (1988) Flowering affected by daylength and temperature in the leafless flowering desert geophyte *Colchicum tunicatum*, its annual life cycle and vegetative propagation. *Botanical Gazette*, 382-390.

Hagenblad, J., Tang, C., Molitor, J., Werner, J., Zhao, K., Zheng, H., Marjoram, P., Weigel, D. & Nordborg, M. (2004) Haplotype structure and phenotypic associations in the chromosomal regions surrounding two *Arabidopsis thaliana* flowering time loci. *Genetics,* **168,** 1627.

Hara, T. (1984) Dynamics of stand structure in plant monocultures. *Journal of Theoretical Biology,* **110,** 223-239.

Harbinson, J. & Foyer, C. (1991) Relationships between the efficiencies of photosystems I and II and stromal redox state in CO_2-free air: evidence for cyclic electron flow in vivo. *Plant physiology,* **97,** 41.

Haritatos, E., Medville, R. & Turgeon, R. (2000) Minor vein structure and sugar transport in Arabidopsis thaliana. *Planta,* **211,** 105-111.

Harper, J. (1967) A Darwinian approach to plant ecology. *The Journal of Ecology,* **55,** 247-270.

Hassell, M. (1975) Density-dependence in single-species populations. *The Journal of Animal Ecology,* **44,** 283-295.

Heichel, G. (1971) Stomatal movements, frequencies, and resistances in two maize varieties differing in photosynthetic capacity. *Journal of Experimental Botany,* **22,** 644.

Helenurm, K. & Barrett, S. (1987) The reproductive biology of boreal forest herbs. . Phenology of flowering and fruiting. *Can. J. Bot,* **65.**

Hensel, L.L., Grbic, V., Baumgarten, D.A. & Bleecker, A.B. (1993) Developmental and Age-Related Processes That Influence the Longevity and Senescence of Photosynthetic Tissues in Arabidopsis. *The Plant Cell,* **5,** 553-564.

Hetherington, A.M. & Woodward, F.I. (2003) The role of stomata in sensing and driving environmental change. *Nature,* **424,** 901-908.

Heursel, J., Ceulemans, R. & Ibrahim, N. (1987) Stomatal Density and Length for Breeding of Evergreen Azaleas (*Rhododendron simsii Planch.*). *Plant Breeding,* **99,** 340-343.

Hoffmann, M.H. (2002) Biogeography of *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae). *Journal of Biogeography,* **29,** 125-134.

Hoffmann, M.H. (2005) Evolution of the reslized climatic niche in the genus *Arabidopsis* (*Brassicaceae*). *Evolution,* **59,** 1425-1436.

Hoffmann, M.H., Bremer, M., Schneider, K., Burger, F., Stolle, E. & Moritz, G. (2003) Flower Visitors in a Natural Population of *Arabidopsis thaliana*. *Plant Biology*, 491-494.

Horton, P. & Bowyer, J. (1990) Chlorophyll fluorescence transients. *Methods in plant biochemistry,* **4,** 259ñ296.

Huang, T., Bohlenius, H., Eriksson, S., Parcy, F. & Nilsson, O. (2005) The mRNA of the Arabidopsis Gene FT Moves from Leaf to Shoot Apex and Induces Flowering. *Science,* **309,** 1694-1696.

Hunt, R. (1982) *Plant growth curves: the functional approach to plant growth analysis*. London.

Hunt, R. & Lloyd, P.S. (1987) Growth and partitioning. *New Phytologist,* **106,** 235-249.

Initiative, A.G. (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature,* **408,** 796-815.

Inouye, B. (2001) Response surface experimental designs for investigating interspecific competition. *Ecology,* **82,** 2696-2706.

IPCC (2007) *Climate Change 2007: the physical science basis: contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge Univ Pr.

Jeje, A. (1985) The flow and dispersion of water in the vascular network of dicotyledonous leaves. *Biorheology,* **22,** 285.

Jiang, H., Li, M., Liang, N., Yan, H., Wei, Y., Xu, X., Liu, J., Xu, Z., Chen, F. & Wu, G. (2007) Molecular cloning and function analysis of the stay green gene in rice. *The Plant Journal,* **0,** ???-???

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R. & Dean, C. (2000) Molecular Analysis of FRIGIDA, a Major Determinant of Natural Variation in Arabidopsis Flowering Time. *Science,* **290,** 344-347.

Johnson, G., Young, A., Scholes, J. & Horton, P. (1993) The dissipation of excess excitation energy in British plant species. *Plant Cell and Environment,* **16,** 673-673.

Jonas, C. & Geber, M. (1999) Variation among populations of *Clarkia unguiculata* (*Onagraceae*) along altitudinal and latitudinal gradients. *American Journal of Botany,* **86,** 333.

Jones, H. (1987) Breeding for stomatal characters. *Stomatal function*, 431ñ443.

Jones, H. (1998) Stomatal control of photosynthesis and transpiration. *Journal of Experimental Botany,* **49,** 387.

Jones, M.E. (1971a) The population genetics of *Arabidopsis thaliana*. I. The breeding system. *Heredity,* **27,** 39-50.

Jones, M.E. (1971b) The population genetics of *Arabidopsis thaliana*. III. The effect of vernalisation. *Heredity,* **27,** 59-72.

Jones, M.E. (1971c) The populations genetics of *Arabidopsis thaliana*. II. Population structure. *Heredity,* **27**.

Juenger, T.E., McKay, J.K., Hausmann, N., Keurentjes, J.J.B., Sen, S., Stowe, K.A., Dawson, T.E., Simms, E.L. & Richards, J.H. (2005) Identification and characterization of QTL underlying whole-plant physiology in *Arabidopsis*

thaliana: δ13C, stomatal conductance and transpiration efficiency. *Plant, Cell and Environment,* **28,** 697-708.

Karssen, C.M., Swan, D.L.C.B.-v.d., Breekland, A.E. & Koornneef, M. (1983) Induction of dormancy during seed development by endogenous abscisic acid : studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. *Planta,* **157,** 158-165.

Keddy, P., Fraser, L.H. & Wisheu, I.C. (1998) A comparative approach to examine competitive response of 48 wetland plant species. *Journal of Vegetation Science,* **9,** 777-786.

Kitajima, M. & Butler, W. (1975) Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. *Biochimica et Biophysica Acta,* **376,** 105.

Klikoff, L. (1966) Competitive response to moisture stress of a winter annual of the Sonoran Desert. *American Midland Naturalist,* **75,** 383-391.

Kolodynska, A. & Pigliucci, M. (2003) Multivariate responses to flooding in Arabidopsis: an experimental evolutionary investigation. *Functional Ecology,* **17,** 131-140.

Koornneef, M., Alonso-Blanco, C. & Vreugdenhil, D. (2004) Naturally occurring genetic variation in *Arabidopsis thaliana. Annual Review of Plant Biology,* **55,** 141-172.

Koornneef, M., Bentsink, L. & Hilhorst, H. (2002) Seed dormancy and germination. *Current Opinion in Plant Biology,* **5,** 33-36.

Koornneef, M., Hanhart, C.J. & Veen, J.H.v.d. (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana. Molecular and general genetics,* **229,** 57-66.

Kooten, O. & Snel, J. (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynthesis Research,* **25,** 147-150.

Krause, G. & Weis, E. (1984) Chlorophyll fluorescence as a tool in plant physiology. *Photosynthesis Research,* **5,** 139-157.

Krause, G. & Weis, E. (1991) Chlorophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Biology,* **42,** 313-349.

Kuittinen, H., Sillanpää , M. & Savolainen, O. (1997) Genetic basis of adaptation: flowering time in *Arabidopsis thaliana. TAG Theoretical and Applied Genetics,* **95,** 573-583.

Kundu, S. & Tigerstedt, P. (1999) Variation in net photosynthesis, stomatal characteristics, leaf area and whole-plant phytomass production among ten provenances of neem (Azadirachta indica). *Tree Physiology,* **19,** 47.

Lagercrantz, U., Putterill, J., Coupland, G. & Lydiate, D. (1996) Comparative mapping in *Arabidopsis and Brassica*, fine scale genome collinearity and congruence of genes controlling flowering time. *The Plant Journal,* **9,** 13-20.

Lake, J. (2004) Gas exchange: new challenges with *Arabidopsis*. *New Phytologist,* **162,** 1-3.

Lambers, H., Pons, T., Chapin III, F., Chapin, I. & Stuart, F. (2008) *Plant physiological ecology*. Springer.

Lambers, H. & Poorter, H. (1987) Inherent variation in growth rate between higher plants: a search for physiological causes and ecological consequences.

Disturbance in Grassland (eds J. van Andel, J.P. Bakker & R.W. Snaydon), pp. 237-251. Dordrecht.

Larcher, W. (1995) *Physiological plant ecology,* Third edition edn. Springer, Berline.

Larkin, J., Marks, M., Nadeau, J. & Sack, F. (1997) Epidermal Cell Fate and Patterning in Leaves. pp. 1109-1120. Am Soc Plant Biol.

Larkin, J., Young, N., Prigge, M. & Marks, M. (1996) The control of trichome spacing and number in *Arabidopsis*. *Development,* **122,** 997.

Law, R. & Watkinson, A. (1987) Response-surface analysis of two-species competition: an experiment on *Phleum arenarium* and *Vulpia fasciculata*. *The Journal of Ecology,* **75,** 871-886.

Lefebvre, S., Lawson, T., Fryer, M., Zakhleniuk, O., Lloyd, J. & Raines, C. (2005) Increased sedoheptulose-1, 7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. *Plant physiology,* **138,** 451.

Levin, M., Lemcoff, J.H., Cohen, S. & Kapulnik, Y. (2007) Low air humidity increases leaf-specific hydraulic conductance of *Arabidopsis thaliana* (L.) Heynh (Brassicaceae). *J. Exp. Bot.*, erm220.

Li, B., Suzuki, J. & Hara, T. (1998) Latitudinal variation in plant size and relative growth rate in Arabidopsis thaliana. *Oecologia,* **115,** 293-301.

Liao, J., Chang, J. & Wang, G. (2005) Stomatal density and gas exchange in six wheat cultivars. *Cereal Research Communications,* **33,** 719-726.

Long, S. & Bernacchi, C. (2003) Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. *Journal of Experimental Botany,* **54,** 2393.

Lu, Z. & Zeiger, E. (1994) Selection for higher yields and heat resistance in Pima cotton has caused genetically determined changes in stomatal conductances. *Physiologia Plantarum,* **92,** 273-278.

Lukowitz, W., Roeder, A., Parmenter, D. & Somerville, C. (2004) A MAPKK Kinase Gene Regulates Extra-Embryonic Cell Fate in Arabidopsis. *Cell,* **116,** 109-119.

Mansfield, T.A., Hetherington, A.M. & Atkinson, C.J. (1990) Some Current Aspects of Stomatal Physiology. *Annual Review of Plant Physiology and Plant Molecular Biology,* **41,** 55-75.

Martinez-Zapater, J.M., Coupland, G., Dean, C. & Koornneef, M. (1994) The Transition to flowering in *Arabidopsis*. *Arabidopsis* (eds E.M. Meyerowitz & C.R. Somerville). Cold Spring Harbor Laboratory Press

.

Martre, P., Morillon, R., Barrieu, F., North, G., Nobel, P. & Chrispeels, M. (2002) Plasma membrane aquaporins play a significant role during recovery from water deficit. *Plant physiology,* **130,** 2101.

Masuda, M. & Washitani, I. (1992) Differentiation of spring emerging and autumn emerging ecotypes in *Galium Mium spurium* L. var. echinospermon. *Oecologia,* **89.**

Matile, P., Hortensteiner, S. & Thomas, H. (1999) Clorophyll degradation. *Annual Review of Plant Physiology,* **50,** 67-95.

Maxwell, K. & Johnson, G. (2000a) Chlorophyll fluorescence--a practical guide. *Journal of Experimental Botany,* **51,** 659.

Maxwell, K. & Johnson, G. (2000b) Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany,* **51,** 659.

Mayer, U., Torres-Ruiz, R.A., Berleth, T., Misera, S. & Jurgens, G. (1991) Mutations affecting body organization in the *Arabidopsis* embryo. *Nature,* **353,** 402-408.

McConnaughay, K.D.M. & Bazzaz, F.A. (1991) Is physical space a soil resource? *Ecology,* **72,** 94-103.

McGraw, J. & Garbutt, K. (1990) The analysis of plant growth in ecological and evolutionary studies. *Trends in Ecology & Evolution,* **5,** 251-254.

McKay, J.K., Richards, J.H. & Mitchell-Olds, T. (2003) Genetics of drought adaptation in *Arabidopsis thaliana*: I. Pleiotropy contributes to genetic correlations among ecological traits. pp. 1137-1151.

McPherson, H. & Slatyer, R. (1973) Mechanisms regulating photosynthesis in *Pennisetum typhoides. Australian Journal of Biological Science,* **26,** 329-339.

Mead, R. (1967) A mathematical model for the estimation of inter-plant competition. *Biometrics,* **23,** 189-205.

Mealor, B. & Hild, A. (2007) Post-invasion evolution of native plant populations: a test of biological resilience. *Oikos,* **116,** 1493-1500.

Mealor, B.A. & Hild, A.L. (2006) Potential selection in native grass populations by exotic invasion. *Journal of Molecular Biology,* **15,** 2291-2300.

Meinke, D. (1994) Seed development in *Arabidopsis thaliana. Arabidopsis* (eds E. Meyerowitz & C. Sommerville), pp. 253-295. Cold Spring Harbor NY.

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D. & Koornneef (1988) Arabidopsis thaliana: a model plant for genome analysis. *Science,* **282,** 662-682.

Meinzer, F. & Grantz, D. (1990) Stomatal and hydraulic conductance in growing sugarcane: stomatal adjustment to water transport capacity*. *Plant, Cell and Environment,* **13,** 383-388.

Meyer, R., Torjek, O., Becher, M. & Altmann, T. (2004) Heterosis of biomass production in Arabidopsis. Establishment during early development. *Plant physiology,* **134,** 1813.

Meyerowitz, E. (1989) Arabidopsis, a useful weed. *Cell,* **56,** 263-269.

Meyerowitz, E. & Pruitt, R.E. (1985) *Arabidopsis thaliana* and plant molecular genetics. *Science,* **229,** 1214-1218.

Meyre, D., Leonardi, A., Brisson, G. & Vartanian, N. (2001) Drought-adaptive mechanisms involved in the escape/tolerance strategies of *Arabidopsis Landsberg erecta* and Columbia ecotypes and their F1 reciprocal progeny. *Journal of Plant Physiology,* **158,** 1145-1152.

Michaels, S.D. & Amasino, R.M. (2001) Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. *The Plant Cell,* **13.**

Miskin, K., Rasmusson, D. & Moss, D. (1972) Inheritance and physiological effects of stomatal frequency in barley. *Crop Science,* **12,** 780.

Mitchell-Olds, T. (1996) Genetic Constraints on Life-History Evolution: Quantitative-Trait Loci Influencing Growth and Flowering in Arabidopsis thaliana. *Evolution,* **50,** 1849-1858.

Mitchell-Olds, T. (2001) Arabidopsis thaliana and its wild relatives: a model system for ecology and evolution. *Trends in Ecology & Evolution,* **16,** 693-700.

Montague, J., Barrett, S. & Eckert, C. (2008) Re-establishment of clinal variation in flowering time among introduced populations of purple loosestrife (Lythrum salicaria, Lythraceae). *Journal of evolutionary biology,* **21,** 234-245.

Moyers, B. & Kane, N. (2010) The genetics of adaptation to novel environments: selection on germination timing in Arabidopsis thaliana. *Molecular Ecology,* **19,** 1270-1272.

Muschak, M., Hoffmann-Benning, S., Fuss, H., Kossmann, J., Willmitzer, L. & Fisahn, J. (1997) Gas exchange and ultrastructural analysis of transgenic potato plants expressing mRNA antisense construct targeted to the cp-fructose-1, 6-bisphosphate phosphatase. *Photosynthetica,* **33,** 455-465.

Muschak, M., Willmitzer, L. & Fisahn, J. (1999) Gas-exchange analysis of chloroplastic fructose-1, 6-bisphosphatase antisense potatoes at different air humidities and at elevated CO 2. *Planta,* **209,** 104-111.

Nadeau, J.A. & Sack, F.D. (2002) Stomatal Development in Arabidopsis.

Napp-Zinn, K. (1961) Über die Bedeutung genetischer Untersuchungen an kältebedürftigen Pflanzen für die Aufklärung von Vernalisationserscheinungen. *Züchter,* **31,** 128-135.

Napp-Zinn, K. (1962) Über die Genetischen Grundlagen des Vernalisationsbedürfnisses bei *Arabidopsis thaliana. Molecular and general genetics,* **93,** 154-163.

Napp-Zinn, K. (1969) *Arabidopsis thaliana* (L.) Heynh. *The induction of flowering, some case histories* (ed. L.T. Evans). Canberra, Australia.

Napp-Zinn, K. (1985) *Arabidopsis thaliana. CRC Handbook of flowering* (ed. A.H. Halevy), pp. 492-503. CRC Press, Boca Raton, Florida.

Nelder, J. (2009) Yield-density relations and Jarvis's lucerne data. *The Journal of Agricultural Science,* **61,** 427-429.

Nooden, L.D. (1984) Integration of soybean pod development and monocarpic senescence. *Physiologia Plantarum,* **62,** 273-284.

Nooden, L.D., Hillsberg, J.W. & Schneider, M.J. (1996) Induction of leaf senescence in Arabidopsis thaliana by long days through a light-dosage effect. *Physiologia Plantarum,* **96,** 491-495.

Nooden, L.D. & Penney, J.P. (2001) Correlative controls of senescence and plant death in Arabidopsis thaliana (Brassicaceae). *Journal of Experimental Botany,* **52,** 2151-2159.

Nord, E. & Lynch, J. (2008) Delayed reproduction in Arabidopsis thaliana improves fitness in soil with suboptimal phosphorus availability. *Plant, Cell & Environment,* **31,** 1432-1441.

Nordborg, M. & Bergelson, J. (1999) The effect of seed and rosette cold treatment on germination and flowering time in some Arabidopsis thaliana (Brassicaceae) ecotypes. *American Journal of Botany,* **86,** 470-475.

Nordborg, M., Hu, T.T., Ishino, Y., Jhaveri, J., Toomajian, C., Zheng, H., Bakker, E., Calabrese, P., Gladstone, J., Goyal, R., Jakobsson, M., Kim, S., Morozov, Y., Padhukasahasram, B., Plagnol, V., Rosenberg, N.A., Shah, C., Wall, J.D., Wang, J., Zhao, K., Kalbfleisch, T., Schulz, V., Kreitman, M. & Bergelson, J. (2005) The Pattern of Polymorphism in Arabidopsis thaliana. *PLoS Biology,* **3,** e196.

Obeso, J. (1993) Selective fruit and seed maturation in Asphodelus albus Miller (Liliaceae). *Oecologia,* **93,** 564-570.

Oksanen, L., Sammul, M. & gi, M. (2006) On the indices of plant-plant competition and their pitfalls. *Oikos,* **112,** 149-155.

Paul-Victor, C., Zust, T., Rees, M., Kliebenstein, D.J. & Turnbull, L.A. (2010) A new method for measuring relative growth rate can uncover the costs of defensive compounds in Arabidopsis thaliana. *New Phytol,* **187,** 1102-1111.

Percy, R.G., Lu, Z., Radin, J.W., Turcotte, E.L. & Zeiger, E. (1996) Inheritance of stomatal conductance in cotton (Gossypium barbadense). *Physiologia Plantarum,* **96,** 389-394.

Pigliucci, M. (2002) Ecology and evolutionary biology of Arabidopsis. *The Arabidopsis book* (eds E. Meyerowitz & C. Somerville), pp. 1-20. ASPB.

Pigliucci, M. (2003) Selection in a model system: ecological genetics of flowering time in *Arabidopsis thaliana. Ecology,* **84,** 1700-1712.

Pigliucci, M. & Schlichting, C.D. (1998) Reaction norms of Arabidopsis. V. Flowering time controls phenotypic architecture in response to nutrient stress. *Journal of evolutionary biology,* **11,** 285-301.

Poethig, R. (1997) Leaf morphogenesis in flowering plants. *The Plant Cell,* **9,** 1077.

Pons, T. & Welschen, R. (2002) Overestimation of respiration rates in commercially available clamp-on leaf chambers. Complications with measurement of net photosynthesis. *Plant Cell and Environment,* **25,** 1367-1372.

Poorter, H. & Garnier, E. (2007) Ecological significance of inherent variation in relative growth rate and its components. *Handbook of Functional Plant Ecology* (eds F.I. Pugnaire & F. Valladares), pp. 81–120.

Potvin, C. & Tousignant, D. (1996) Evolutionary consequences of simulated golbal change: genetic adaptation or adaptative phenotypic plasticity. *Oecologia,* **108,** 683-693.

Poulson, M., Boeger, M. & Donahue, R. (2006) Response of photosynthesis to high light and drought for Arabidopsis thaliana grown under a UV-B enhanced light regime. *Photosynthesis Research,* **90,** 79-90.

Pugnaire, F.I. & Luque, M.T. (2001) Changes in plant interactions along a gradient of environmental stress. *Oikos,* **93,** 42-49.

Quick, W. & Horton, P. (1984) Studies on the induction of chlorophyll fluorescence in barley protoplasts. II. Resolution of fluorescence quenching by redox state and the transthylakoid pH gradient. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* **220,** 371-382.

Radin, J.W. (1984) Stomatal Responses to Water Stress and to Abscisic Acid in Phosphorus-Deficient Cotton Plants. pp. 392-394.

Radin, J.W., Lu, Z., Percy, R.G. & Zeiger, E. (1994) Genetic variability for stomatal conductance in Pima cotton and its relation to improvements of heat adaptatiom. *Proc. Natl. Acad. Sci.,* **91,** 7217-7221.

Rasband, W. (2007) ImageJ, US National Institutes of Health, Bethesda, Maryland, USA. *Website http://rsb. info. nih. gov/ij/[accessed March 2007].*

Rascher, U., Liebig, M. & Luttge, U. (2000) Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. *Plant Cell and Environment,* **23,** 1397-1405.

Ratcliffe, D. (1961) Adaptation to habitat in a group of annual plants. *Journal of Ecology,* **49,** 187-203.

Raz, V., Bergervoet, J.H.W. & Koornneef, M. (2001) Sequential steps for developmental arrest in Arabidopsis seed. *Development,* **128,** 243-253.

Redei, G.P. (1975) *Arabidopsis* as a genetic tool. *Annual Review of Genetics,* **9,** 111-127.

Rosenqvist, E. & van Kooten, O. (2003) Chlorophyll fluorescence: a general description and nomenclature. *Practical applications of chlorophyll fluorescence in plant biology,* 31-77.

Ross, M.A. & Harper, J.L. (1972) Occupation of biological space during seedling establishment. *Journal of Ecology,* **60,** 77-88.

Roth-Nebelsick, A., Uhl, D., Mosbrugger, V. & Kerp, H. (2001) Evolution and function of leaf venation architecture: a review. *Annals of Botany,* **87,** 553.

Roy, B., Stanton, M. & Eppley, S. (1999) Effects of environmental stress on leaf hair density and consequences for selection. *Journal of evolutionary biology,* **12,** 1089-1103.

Samson, G., Prášil, O. & Yaakoubd, B. (1999) Photochemical and thermal phases of chlorophyll a fluorescence. *Photosynthetica,* **37,** 163-182.

Sandring, S., Riihimäki, M., Savolainen, O. & ≈gren, J. (2007) Selection on flowering time and floral display in an alpine and a lowland population of Arabidopsis lyrata. *Journal of evolutionary biology,* **20,** 558-567.

Schlüter, U., Köpke, D., Altmann, T. & Müssig, C. (2002) Analysis of carbohydrate metabolism of CPD antisense plants and the brassinosteroid-deficient cbb1 mutant. *Plant, Cell & Environment,* **25,** 783-791.

Schlüter, U., Muschak, M., Berger, D. & Altmann, T. (2003) Photosynthetic performance of an Arabidopsis mutant with elevated stomatal density (sdd1-1) under different light regimes. *J. Exp. Bot.,* **54,** 867-874.

Schmitt, J., McCormac, A.C. & Smith, H. (1995) A test of the adaptive plasticity hypothesis using transgenic and mutant plants disabled in phytochrome-mediated elongation responses to neighbors. *The American Naturalist,* **146,** 937-953.

Schreiber, U. (1986) Detection of rapid induction kinetics with a new type of high-frequency modulated chlorophyll fluorometer. *Photosynthesis Research,* **9,** 261-272.

Schreiber, U., Schliwa, U. & Bilger, W. (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynthesis Research,* **10,** 51-62.

Schulze, E.D. (1986) Carbon Dioxide and Water Vapor Exchange in Response to Drought in the Atmosphere and in the Soil. pp. 247-274.

Sharbel, T.F., Haubold, B. & Mitchell-Olds, T. (2000) Genetic isolation by distance in Arabidopsis thaliana: biogeography and postglacial colonization of Europe. *Molecular Ecology,* **9,** 2109-2118.

Sharkey, T. (1985) Photosynthesis in intact leaves of C 3 plants: physics, physiology and rate limitations. *The Botanical Review,* **51,** 53-105.

Sharkey, T., Bernacchi, C., Farquhar, G. & Singsaas, E. (2007) Fitting photosynthetic carbon dioxide response curves for C3 leaves. *Plant Cell and Environment,* **30,** 1035.

Sharp, R. & Davies, W. (1979) Solute regulation and growth by roots and shoots of water-stressed maize plants. *Planta,* **147,** 43-49.

Sharp, R. & LeNoble, M. (2002) ABA, ethylene and the control of shoot and root growth under water stress. *Journal of Experimental Botany,* **53,** 33.

Sharp, R., Poroyko, V., Hejlek, L., Spollen, W., Springer, G., Bohnert, H. & Nguyen, H. (2004) Root growth maintenance during water deficits: physiology to functional genomics. *Journal of Experimental Botany,* **55,** 2343.

Sharp, R.E. & Davies, W.J. (1985) Root growth and water uptake by maize plants in drying soil. *Journal of Experimental Botany,* **36,** 1441-1456.

Sharpe, P. & Dent, J. (2009) The determination and economic analysis of relationships between plant population and yield of main crop potatoes. *The Journal of Agricultural Science,* **70,** 123-129.

Sheldon, C.C., Rouse, D.T., Finnegan, E.J., Peacock, W.J. & Dennis, E.S. (2000) The molecular basis of vernalization: The central role of FLOWERING LOCUS C (FLC). *Proceedings of the National Academy of Sciences of the United States of America,* **97,** 3753-3758.

Shimizu, K.K., Cork, J.M., Caicedo, A.L., Mays, C.A., Moore, R.C., Olsen, K.M., Ruzsa, S., Coop, G., Bustamante, C.D., Awadalla, P. & Purugganan, M.D. (2004) Darwinian Selection on a Selfing Locus. *Science,* **306,** 2081-2084.

Shindo, C., Bernasconi, G. & Hardtke, C.S. (2007) Natural Genetic Variation in Arabidopsis: Tools, Traits and Prospects for Evolutionary Ecology. *Annals of Botany,* 1-12.

Silvertown, J. & Charlesworth, D. (2001) *Introduction to plant population biology.* Wiley-Blackwell.

Simpson, G.G. & Dean, C. (2002a) Arabidopsis, the rosetta ston of flowering time? *Science,* **296,** 285-289.

Simpson, G.G. & Dean, C. (2002b) Arabidopsis, the rosetta stone of flowering time? *Science,* **296.**

Sinclair, T., Tanner, C. & Bennett, J. (1984) Water-use efficiency in crop production. *BioScience,* **34,** 36-40.

Stinchcombe, J., Dorn, L. & Schmitt, J. (2004a) Flowering time plasticity in Arabidopsis thaliana: a reanalysis of Westerman & Lawrence (1970). *Journal of evolutionary biology,* **17,** 197-207.

Stinchcombe, J.R., Dorn, L.A. & Schmitt, J. (2004b) Flowering time plasticity in *Arabidopsis thaliana*: a reanalysis of Westerman & Lawrence (1970). *Journal of evolutionary biology,* **17.**

Sun, J., Okita, T. & Edwards, G. (1999) Modification of carbon partitioning, photosynthetic capacity, and O2 sensitivity in Arabidopsis plants with low ADP-glucose pyrophosphorylase activity. *Plant physiology,* **119,** 267.

Taiz, L. & Zeiger, E. (2006) *Plant Physiology,* Fourth edn. Sinauers eds., Sunderland.

Team, R.D.C. (2010) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Telfer, A., Bollman, K. & Poethig, R. (1997) Phase change and the regulation of trichome distribution in Arabidopsis thaliana. *Development,* **124,** 645.

Telfer, A. & Poethig, R.S. (1994) Leaf Development in Arabidopsis. *Arabidopsis* (eds E.M. Meyerowitz & C.R. Somerville). Cold Spring Harbor Laboratory Press.

Tholen, D., Voesenek, L. & Poorter, H. (2004) Ethylene insensitivity does not increase leaf area or relative growth rate in Arabidopsis, Nicotiana tabacum, and Petunia x hybrida. *Plant physiology,* **134,** 1803.

Thomas, S.C. & Bazzaz, F.A. (1993) The genetic component in plant size hierarchies: norms of reaction to density in a polygonum species. *Ecological Monographs,* **63,** 231-249.

Tilman, D. (1980) Resources: a graphical-mechanistic approach to competition and predation. *American Naturalist,* **116,** 362-393.

Tilman, D. (1982) *Resource competition and community structure*. Princeton Univ Press.

Tilman, D. (1988) *Plant Strategies and the Dynamics and Structure of Plant Communities*. Princeton University Press.

Tocquin, P. & Perilleux, C. (2004) Design of a versatile device for measuring whole plant gas exchanges in Arabidopsis thaliana. *New Phytologist,* **162,** 223-229.

Tsuge, T., Tsukaya, H. & Uchimiya, H. (1996) Two independent and polarized processes of cell elongation regulate leaf blade expansion in Arabidopsis thaliana (L.) Heynh. *Development,* **122,** 1589.

Tsukaya, H. (1995) Developmental genetics of leaf morphogenesis in dicotyledonous plants. *Journal of Plant Research,* **108,** 407-416.

Tsukaya, H., Shoda, K., Kim, G. & Uchimiya, H. (2000) Heteroblasty in Arabidopsis thaliana (L.) Heynh. *Planta,* **210,** 536-542.

Tyree, M.T. (2007) Water relations and hydraulic architecture. *Handbook of Functional Ecology*
(eds F. Pugnaire & F. Valladares). CRC Press.

Valladares, F. & Pearcy, R.W. (1997) Interactions between water stress, sun-shade acclimation, heat tolerance and photoinhibition in the sclerophyll *Heteromeles arbutifolia*. *Plant, Cell and Environment,* **20,** 25-36.

Valladares, F., Sanchez-Gomez, D. & Zavala, M.A. (2006) Quantitative estimation of phenotypic plasticity: bridging the gap between the evolutionary concept and its ecological applications. pp. 1103-1116.

van den Honert, T. (1948) Water transport as a catenary process. *Discussions of the Faraday Society,* **3,** 146-153.

Van Der Kooij, T. & De Kok, L. (1996) Impact of elevated CO~ 2 on growth and development of Arabidopsis thaliana L. *PHYTON-HORN-,* **36,** 173-184.

Van Der Weele, C., Spollen, W., Sharp, R. & Baskin, T. (2000) Growth of Arabidopsis thaliana seedlings under water deficit studied by control of water potential in nutrient-agar media. *Journal of Experimental Botany,* **51,** 1555.

Vandermeer, J. (1984) Plant competition and the yield-density relationship*. *Journal of Theoretical Biology,* **109,** 393-399.

Vernon, A. & Allison, J. (1963) A method of calculating net assimilation rate.

Von Groll, U., Berger, D. & Altmann, T. (2002) The Subtilisin-Like Serine Protease SDD1 Mediates Cell-to-Cell Signaling during Arabidopsis Stomatal Development. pp. 1527-1539.

Vu, J., Allen Jr, L. & Bowes, G. (1984) Dark/light modulation of ribulose bisphosphate carboxylase activity in plants from different photosynthetic categories. *Plant physiology,* **76,** 843.

Walton, P. (1974) The genetics of stomatal length and frequency in clones of Bromus inermis and the relationships between these traits and yield. *Can. J. Pl. Sci,* **54,** 749-754.

Warren, C. (2007) Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO_2 transfer. *Journal of Experimental Botany*.

Watkinson, A.R. (1980) Density-dependence in single-species populations of plants. *Journal of Theoretical Biology,* **83,** 345-357.

Weiner, J., Stoll, P., Muller-Landau, H. & Jasentuliyana, A. (2001) The effects of density, spatial pattern, and competitive symmetry on size variation in simulated plant populations. *American Naturalist,* **158,** 438-450.

Weiner, J. & Thomas, S.C. (1992) Competition and allometry in a three species of annual plants. *Ecology,* **73,** 648-656.

Weining, C., Johnston, J., German, Z.M. & Demink, L.M. (2006) Local and global cost of adaptative plasticity to density in Arabidopsis thaliana. *The American Naturalist,* **167,** 826-836.

Werner, J., Borevitz, J., Warthmann, N., Trainer, G., Ecker, J., Chory, J. & Weigel, D. (2005) Quantitative trait locus mapping and DNA array hybridization identify an FLM deletion as a cause for natural flowering-time variation. *Proceedings of the National Academy of Sciences,* **102,** 2460.

Werner, P.A. (1975) Predictions of fate from rosette size in teasel (*Dipsacus fullonum* L.). *Oecologia,* **20,** 197-201.

Westerman, J.M. (1970a) Genotype-environment interaction and developmental regulation in *Arabidopsis thaliana*. II. Inbred lines; analysis. *Heredity,* **26,** 93-106.

Westerman, J.M. (1970b) Genotype-environment interaction and developmental regulation in *Arabidopsis thaliana*. III. Inbred lines; analysis of response to photoperiod. *Heredity,* **26,** 373-382.

Westerman, J.M. (1970c) Genotype-environment interaction and developmental regulation in *Arabidopsis thaliana*. IV. Wild material: analysis. *Heredity,* **26,** 383-395.

Westerman, J.M. & Lawrence, M.J. (1970) Genotype-environment interaction and developmental regulation in *Arabidopsis thaliana*. I. Inbred lines; description. *Heredity,* **25.**

Westgate, M. & Boyer, J. (1985) Osmotic adjustment and the inhibition of leaf, root, stem and silk growth at low water potentials in maize. *Planta,* **164,** 540-549.

Willey, R. & Heath, S. (1969) The quantitative relationships between plant population and crop yield. *Advances in Agronomy,* **21,** 281-321.

Willmer, C. & Fricker, M. (1996) *Stomata*. Chapman & Hall, London.

Wilson, J.B. (1988a) A review of evidence on the control of shoot:root ratio, in relation to models. *Annals of Botany* **61,** 433-449.

Wilson, J.B. (1988b) Shoot competition and root competition *Journal of Applied Ecology,* **25,** 279-296.

Wilson, S., D. & Tilman, D. (1993) Plant competition and resource availability in response to disturbance and fertilization. *Ecology,* **74,** 599-611.

Woodward, F.I. (1987) Stomatal numbers are sensitive to increases in CO_2 from pre-industrial levels. *Nature,* **327,** 617-618.

Woodward, F.I. (1993) Plant responses to past concentrations of CO_2. *Plant Ecology,* **104-105,** 145-155.

Woodward, F.I. & Bazzaz, F.A. (1988) The Responses of Stomatal Density to CO_2 Partial Pressure. *Journal of Experimental Botany,* **39,** 1771-1781.

Woodward, F.I. & Kelly, C.K. (1995) The influence of CO2 concentration on stomatal density. *New Phytologist,* **131,** 311-327.

Woodward, F.I., Lake, J.A. & Quick, W.P. (2002) Stomatal development and CO_2: ecological consequences. pp. 477-484.

Xie, X., Wang, Y., Williamson, L., Holroyd, G., Tagliavia, C., Murchie, E., Theobald, J., Knight, M., Davies, W. & Leyser, H. (2006) The identification of genes involved in the stomatal response to reduced atmospheric relative humidity. *Current Biology,* **16,** 882-887.

Yang, M. & Sack, F.D. (1995) The too many mouths and four lips Mutations Affect Stomatal Production in Arabidopsis. *Plant Cell,* **7,** 2227-2239.

Ye, Z. (2007) A new model for relationship between light intensity and the rate of photosynthesis in Oryza sativa. *Photosynthetica,* **45,** 637ñ640.

Yoshida , T., Dale, N. & Rasmusson, D. (1975) Effect of stomatal frequency in barley on photosynthesis and transpiration. *Bull. Kyushu Agrie. Expt. Sta,* **18.**

Yousufzai, M., Siddiqui, K. & Soomro, A. (2009) Flag leaf stomatal frequency and its interrelationship with yield and yield components in wheat (*Triticum aestivum* L.). *Pak. J. Bot,* **41,** 663-666.

Yu, Q. (2001) Can physiological and anatomical characters be used for selecting high yielding hybrid aspen clones? *Silva Fennica,* **35,** 137-146.

Zhang, H. & Forde, B.G. (1998) An Arabidopsis MADS Box Gene That Controls Nutrient-Induced Changes in Root Architecture. pp. 407-409.

Zhang, J. & Lechowicz, M.J. (1994) Correlation between time of flowering and phenotypic plasticity in Arabidopsis thaliana (brassicaceae). *American Journal of Botany,* **81,** 1336-1342.

Zhao, L. & Sack, F.D. (1999) Ultrastructure of stomatal development in Arabidopsis (Brassicaceae) leaves. pp. 929-939.

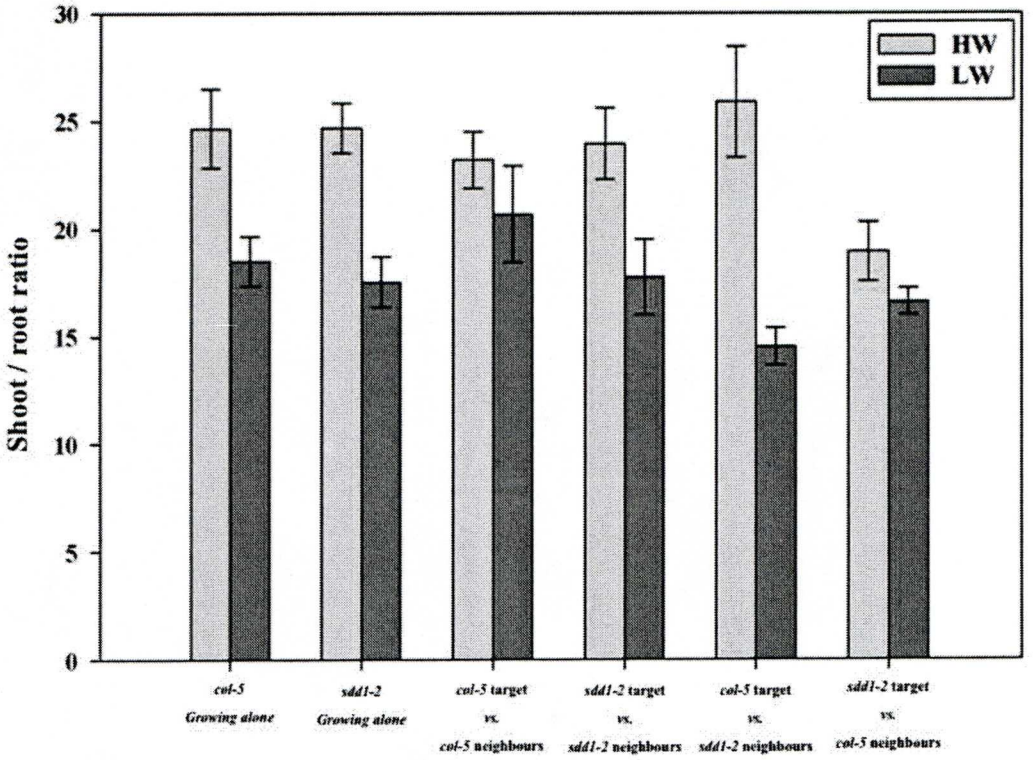

Figure 6. 2. Above- to belowground ratios of biomass at time of flowering for both genotypes in monogenotypic stand and mixture.The Figure includes data taken from Chapter 4, that describe the biomass partitioning of isolated plants.

Table 6. 3. Two-factor ANOVA of biomass ratios for genotypes growing alone and in intra- and inter-genotypic competition under HW or LW. Water regime: high water (HW) and water-limited (LW) conditions. Genotypes were *sdd1-2* and *col-5*. The term 'competition refers to the density /mixture combination were plants growing in intra-genotypic competition and plants growing in inter-genotypic competition.

Source of variation	df	SS	F value	P
Water regime	1	1096.0	1096.0	<0.01
Genotype	1	54.4	54.4	0.177
Competition	2	170.5	85.3	0.059
Water x genotype	1	29.5	29.5	0.319
Water x competition	2	28.9	14.4	0.614
Genotype x competition	2	40.2	20.1	0.507
Water x genotype x competition	2	281.5	140.7	<0.05
Residuals	120	3537.4	29.5	